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LIGHT AND ELECTRON MICROSCOPICAL STUDIES OF THE EFFECT OF CRYOSURGERY ON SKELETAL MUSCLE AND PERIPHERAL NERVE OF THE GUINEA PIG AND HORSE SKIN.

by

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VOLUME 1

I, the undersigned, hereby declare that this thesis embodies the results of my own work and that it has been composed by myself.

A.J.G. Aldeen.

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ERRATA

Page	Line	Wrong	Correct
98	23	Kaldern and Gihila	Kaldern and Gilula
99	-	Mayblasts	Myotubes
112	15	The endothelial	The endoneural
113	2	were open	were closed
114	1	The endothelial	The endoneural
114	11	With Loyez's	With Mason's
114	26	Fig. 5.22	Fig. 5.21
116	26	with silver	with silver stain
119	25	Schwann cell nuclei	Schwann cells
120	1	Schwann cell nuclei	Schwann cells
120	17	Schwann cell nuclei	Schwann cells
120	26	Fig. 5.51	Fig. 5.52
146	-	distribution	disruption
157	24	were less consolidated	were less consolidated (Fig. 7.17a)
167	2	Ranson	Ronson
184	7	Fig. 8.32	Fig. 8.35
209	2	Matolsty and Parakka	Matolsty and Parakkal
220	16	Metaplasma	Metaplasia
233	26	J.A.V.M.A.	J.A.M.A.
247	28	Squiet and Johnson	Squier and Johnson
250	16	Warayma	Wakayma
148	10	Improved vascular and energy supply	Severe drop in energy supply.

ERRATA

References omitted:

CARL, M.P. (1962) The pathologic reaction of skeletal muscle. in "Diseases of Muscle" 2nd edition. pub. Henry Kimpton, London.

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CHAPTER 1

CRYOSURGERY: AN INTRODUCTION, HISTORICAL BACKGROUND AND TECHNIQUES.

A Introduction

When living cells or tissues are reduced to very low temperatures cell and tissue destruction can occur. The controlled destruction of diseased tissue in clinical patients by the application of cold is termed - CRYOSURGERY - in which Kryos in ancient Greek means frost.

Cryosurgery is a clinical technique applied to diseased animal tissues and particularly to malignant tumours. The diseased tissues are subjected to direct sub-zero temperatures. Following treatment the tissues necrose and are sloughed. The subsequent healing is by secondary intention. Cryosurgery is being used increasingly in human and veterinary practice and many questions remain to be answered about the destructive power of cryosurgery on living tissue and the fate of non-diseased tissue adjacent to a cryosurgical lesion.

There is a considerable amount of recorded data concerning tissue damage, death and repair following several types of injury including the effect of freezing. This present study was designed to observe the effect of controlled freezing on living tissues to cryogenic or cryo-destructive temperatures, with particular reference to the healing processes involved and the degree of tissue repair in different areas of the cryo-lesion.

Skeletal muscle and peripheral nerves are frequently frozen either intentionally or inadvertently, during cryo-therapy of deeper lesions and it was considered important to examine the effects of cold injury in these tissues.

The investigation is related to the histopathology of the tissue changes during destruction, repair and regeneration employing light microscopy and electron microscopy techniques.

Cryosurgery has been employed with success in the treatment of lesions of the skin and adnexa in man and some species of domestic animals but comparable results have not been achieved in treating benign fibrous skin tumours in the horse (Borthwick, 1970). There was a high percentage of recurrence of neoplasms in cryosurgically treated horse skin. The clinical problems of the surgery of horse skin stimulated the third part of the investigations reported in this thesis.

B Historical background

The therapeutic value of cold applications has been recognised for many centuries. In 2500 B.C. cold applications were used by ancient Egyptians as a palliative treatment in different types of bone fractures (Edwin, 1930; Sweet Roland, 1968). Homer recognised the benefit of cold application locally in the treatment of chest wounds (Tytus, 1968) and Hippocrates used local cold applications to control haemorrhage and to reduce swelling (Tytus, 1968).

The Arabian physician, Ibn Sina in 980 A.D. had used cold applications in many medical situations, such as headache, local trauma, haemorrhage and for reducing high body temperature; while its use in the treatment of dental cases, especially toothache formed a separate chapter under Local Anaesthesia, in his famous book - "The Law of Medicine".

Cold was again advocated as a local anaesthetic by an unknown Anglo-Saxon monk in 1050 A.D. (Tytus, 1968). After the battle of Preuss-Eylane in 1807, Napoleon's Surgeon-General, Baron Dominique Jean Larry, indicated that amputation of limbs could be performed painlessly after exposure to cold.

In 1845 James Arnott of Brighton and London was the first to pioneer the controlled use of refrigeration in medicine. He pursued the therapeutic effect of local freezing and was the first to apply freezing techniques in the treatment of cancer and other diseases (Arnott, 1845, 1846, 1851). Arnott achieved temperatures as low as $-12^{o}C$, using cold salt water, circulating in a special container and he also employed cold as a local anaesthetic agent.

John Hughes Bennett in 1849 also used cold application in the treatment of different types of superficial tumours. In 1865 Cook advocated cold applications for the treatment of cancer.

By 1936 reduced temperatures were being used in the treatment of carcinomas (Fay and Henney, 1938).

Fay and Henney (1938) used a hollow capsular device connected to a continuously circulating mixture of water and ice over a period of several weeks, the temperature achieved was -36°F- -48°F. In 1940 Fay reported a study on prolonged human refrigeration; these studies were of special importance as at that time heart surgery was well recognised.

In 1959 Rowbotham and his colleagues, achieved a temperature of -20°C, when they designed a cooling cannula in which 95% alcohol was cooled by passing it through a mixture of solid carbon dioxide (dry ice) and acetone. This instrument was used for the local treatment of inoperable glioma.

The first precision cryosurgical system was designed by Cooper and his team in 1961, using liquid nitrogen as the cryogenic material, and the temperature achieved by the probe tip was as low as -196°C. Since the 1960's many new sophisticated cryosurgical machines have been produced commercially, mainly employing either liquid nitrogen or nitrous oxide as the refrigerants.

During the 1970's the therapeutic use of cryosurgery had increased and has been shown to be useful in the treatment of many different types of tumours, in gynaecology, ophthalmology, neurology and ear, nose and throat practice (Holden, 1975). Although cryotherapy was originally used in man, much of the experimental work was done on domestic and laboratory animals. Cryosurgery in veterinary practice is relatively new, as it was not until the late

1960's that there was a surge of interest in the use of cryosurgical techniques. Cold applications were used for freeze branding in domestic animals (Farrell, 1966; Farrell, Koger and Windward, 1966; Macpherson and Penner, 1967; Farrell, George and Thomas, 1969). Cold applications have also been used in laboratory animals (Farrell and Shirley, 1973).

By the early 1970's therapeutic cryosurgery was being employed in veterinary surgery for the treatment of diseases, such as perianal sinuses, adenomas, ulcerated papillomas, histocytomas and abnormal granulation tissue of the skin of small domestic animals (Borthwick, 1970, 1971, 1972; Lane, 1974; Lane and Burch, 1975). More recently cryosurgery has been used to treat sarcoids and other lesions of the skin of horses (Joyce, 1975, 1976; Farris, Fraundfelder and Finch, 1975; Farris, Fraundfelder and Mason, 1975); in occular squamous cell carcinomas of cattle (Farris and Fraundfelder, 1976), for other cutaneous neoplastic and non-neoplastic lesions in the skin of large animals (Farris et al., 1975; Twidle, 1977) and for neurectomy in horses (Lloyd, 1977).

C Cryogens and instrumentation

Originally the application of cold as a therapeutic device was entirely dependant on the use of cold water, cooled to even lower temperatures by several different methods. Many modifications were introduced to these systems to circulate and cool agents other than water.

Other methods of achieving low temperatures, for example

solid carbon dioxide (dry ice) had been introduced for the treatment of superficial skin lesions (Carpenter, 1943). In the 1960's cryogenic engineering benefited from space exploration technology and several types of cryosurgical machines employing different coolants have been introduced to clinical medicine.

At the present time, the coolants or refrigerants used in cryosurgery are:-

a) Liquid nitrogen (N_2): A clear, chemically inert, non-inflammable, non-toxic liquid whose boiling point at atmospheric pressure is -195·8°C.

b) Nitrous oxide (N_2O): This is a convenient refrigerant when pressurised to a liquid state in special cylinders. It is used as a refrigerant in cryo-applicators employing the Joule-Thomson phenomenon. It is usually used in human and veterinary medical practice as a general anaesthetic in its gaseous form.

c) Solid carbon dioxide (CO_2): Carbon dioxide is sublimated to produce solid sticks whose temperature reaches -78·5°C. It is non-inflammable and odourless. Precautions are usually taken during application to prevent high concentration of CO_2 in the atmosphere as it may have a deleterious effect on the respiratory system.

d) "Freon" 22: A halogenated hydrocarbon usually stored and supplied in steel cylinders as a liquid under its own vapour pressure. It is colourless, non-inflammable and relatively non-toxic. Its boiling point is

relatively high (-10°C), so it has limited therapeutic value.

The methods of producing low temperatures depend on the physical properties of the refrigerant and the design of the cryosurgical instrument.

The most important practical methods of achieving low temperatures in cryosurgery are:-

a) Change of phase:

This is a physical method, which harnesses the loss of latent heat of fusion or vaporisation when solid or liquid matter changes respectively to liquid or gas. The calories required for this phenomenon are extracted from the animals' tissues which reach sub-zero temperatures and loose their vitality. Examples are solid carbon dioxide - solid to gas - and liquid nitrogen - liquid to gas.

Liquid nitrogen is the most popular coolant because of its low boiling point and is readily available. Temperatures of around -195·8°C can be maintained by direct flow to the probe tip or by fine droplet spraying techniques.

There are several types of cryosurgical machines for use with liquid nitrogen, which all employ the same basic principles. They consist of a sealed vacuum-insulated container from which liquid nitrogen is delivered by its own gas pressure along a withdrawal tube to an insulated hand-probe, which may be attached to the closed tips or to fine spray nozzles.

The first cryosurgical unit using liquid nitrogen capable of discrete, controlled application was developed by Dr. I.S. Cooper and the Linde Division of the Union Carbide Corporation in 1961 and many improvements have subsequently been introduced.

Most of the earlier cryosurgery units depended on an electric supply to vapourise the liquid nitrogen and control its flow, and to re-heat the probe tip to facilitate its removal from frozen tissue. Recently cryo-units have been developed which are independant of electrical heating and as these are portable they are of special value in veterinary practice.

The Frigitronics CE8 cryosurgical system was employed in the present study, and it allowed the temperature of the tissue to be reduced to the boiling point of liquid nitrogen by either direct contact with the cryoprobes or by a spray of a mixture of liquid nitrogen droplets and cold nitrogen gas.

The change of phase of solid carbon dioxide has been used in general cryosurgery but mainly for skin disorders and very small tumours.

b) Expansion of compressed gas through a narrow orifice, the Joule-Thomson effect: This phenomenon occurs when a pressurised gas is allowed to escape through a narrow orifice so the temperature at the orifice is reduced and heat will be extracted from the tissue. Cryosurgical units employing this effect are designed to use nitrous oxide or carbon dioxide gas.

Nitrous oxide is preferred because of its availability and better freezing capability.

c) Thermo-electric method: By using the physical properties of thermocouples, cold can be produced but it is difficult to achieve temperatures of below -20°C with the units produced to date. These temperatures are not very effective in clinical application.

In veterinary practice, several types of cryosurgical units using liquid nitrogen have been used successfully, such as the Rubinstein NCPU2 (STP), (Spembley Limited, Andover, England), Linde-Cooper CE-2A (Frigitronics, Connecticut, United States of America) and recently the Frigitronics DFs 30 (Spembley Limited, Andover, England), Frigitronics CE8 (Frigitronics, Connecticut, United States of America) and "Nitrospray" (Arnolds Veterinary Products, Reading, England). Units employing nitrous oxide of the Amoils-Joule-Thomson type are also commonly used (Garamy, 1968; Borthwick, 1970, 1972; Lane, 1974; Holden, 1975).

Tissue temperature recorders:

Tissue temperature recorders are invaluable in cryosurgery to monitor the changes in temperature in the frozen tissues to ensure that all the diseased tissue is frozen to a fatal level and at the same time to prevent over freezing of adjacent healthy tissue. These units consist of a series of thermocouples mounted in special hypodermic needles, this minimises their traumatic effect. The e.m.f. out-put of the thermocouples activates a galvanometer

which gives the readings on a temperature scale.

In some of the more advanced cryosurgical units the tissue temperature recording device can be pre-set to control the freezing automatically, but more usually the recorder is self contained in a separate console, and the freezing is controlled by the surgeon.

The thermocouples are inserted at different levels in the diseased tissue and in adjacent surrounding healthy tissue before treatment starts.

D Cell injury and biological consequences of cryosurgery

The principles involved in cell destruction are mainly a direct, an indirect and possibly a latent phase (Holden, 1973).

i) Direct phase of cellular destruction

Water is the major component of a living system and is very important for biological, chemical and physiological functions of the cells. The removal of water from such a system is a lethal and destructive factor.

The removal of water as ice in a biological system is not an isothermal event, the change of phase being completed over a range of temperatures rather than at the fixed temperature at which ice is formed -0^{o}C. A fluid phase will remain until all particles of the system pass through the eutectic temperature and the eutectic temperature of sodium chloride is one of the most important factors in the process, because this salt is the most abundant electrolyte in mammalian tissue. The concentration

of sodium chloride and other salts, will increase continuously as the formation of ice proceeds, sodium chloride achieving levels of 200 gm/litre and the concentration becomes very toxic to the cell as the eutectic temperature of -21·2°C is reached (Mazur, 1963; Rinfret, 1968; Farrant and Morris, 1972; Silvaris, Caravalho, Tascano and Huggins, 1975).

Lovelock (1953) showed that increased salt concentrations can interact with the cell membrane, altering the normal function and the characteristics of the membrane's permiability. As dehydration proceeds through extra-cellular ice formation, the balance of the buffer system will alter, and induce a change in the hydrogen ion concentration (pH) of the cellular medium, which is potentially very damaging to the cell system (Rinfret 1968). Therefore the formation of ice crystals in tissues subjected to freezing is practically important. As freezing takes place, ice crystals may form either exclusively in the inter cellular spaces, or both inter and intra-cellularly. The mechanism of ice formation is greatly dependant on the rate of freezing so if cells are cooled sufficiently slowly, they will never become super-cooled, but their fluids will be maintained virtually in continuous equilibrium with the ice formed outside the cell. The equilibrium will be maintained by continuous dehydration, as water is shunted from the cell to the inter cellular spaces, which will lead to cell contraction (Towill and Mazur, 1976). As cells shrink to below their minimum

volume, a pressure gradient is produced across the cell membrane, which causes irreversable damage to the cell membrane (Merymann, 1968).

In fast freezing, water is not able to leave the cells sufficiently rapidly to maintain a pressure equilibrium status, so the cells become increasingly supercooled. Supercooled water is unstable and will turn to ice inside the cells (Mazur, 1961, 1965; Menz and Luyet, 1961; Rinfret, 1968; Litvan, 1972). In fast freezing some of the cells within the frozen tissue may reach a temperature of $-20^{o}C$ without actually becoming frozen; in such instances, these cells reach a state of being supercooled. During subsequent slow thawing these cells reach a temperature of less than $-20^{o}C$ when ice crystals appear due to the latent heat of crystalization. In general, the critical temperature of cell death is variable, ranging from $0^{o}C$ to $-20^{o}C$.

During the thaw stage, the tiny ice crystals formed inside the cells during fast freezing will increase in size and so increasing the damaging effect in the cells. Small ice crystals are not very harmful to cells (Anderson, Green and Mazur, 1966; Mazur and Schmidt, 1968). Small ice crystals have a higher surface energy than the larger ice crystals formed by slow freezing. As the tissue begins to thaw slowly, the phenomenon of recrystalization occurs and the small ice crystals grow or agglemerate. In turn, these larger ice crystals cause more damage to the cells (Moor, 1964). Another direct lethal factor created

by cold application is the denaturation of cell proteins and the lipoproteins mainly found in the cell membrane. Lipoproteins are also found in the nuclear membranes, mitochondrial membranes etc. These lipoproteins will show denaturation which is catastrophic to the cells (Lovelock, 1957; Levitt and Dear, 1970). The last possible direct injury to the cells occurs in cells near the periphery of a lesion, they absorb water from thawed extracellular ice crystals and rupture (Leopard and Poswillow, 1974). During the early stages of freezing, cells may be irreversably injured by "cellular shock" (Borthwick, 1970; Farrant and Morris, 1972).

ii) Indirect phase of cellular destruction

In addition to the immediate cellular changes during and directly after cold treatment, delayed cellular injury appears to play an important part in cell destruction. During the thawing stages, blood vessels within the frozen mass show early degenerative changes, mainly within the endothelial cells of the capillaries and small blood vessels. These endothelial cells become swollen and the degenerative changes weaken the vessel walls, which in turn leads to an increase in vascular permiability and blood stasis will ensue (Walder, 1968; Whittaker, 1972; Rabb, Renaud, Brandt and Wittn, 1974). Swollen, degenerated endothelial cells increases the likelihood of microthrombi plugging the lumen of the blood vessels, causing ischaemic infarction (Fraser and Gill, 1967; Walder, 1968; Gill, Long, Fraser and Lee, 1970; Leopard and Poswillow, 1974; Rabb et al., 1974).

Large blood vessels affected by a freezing episode may resume normal function soon after thawing although their endothelial cells show typical degenerative changes and exhibit increased permiability (Gage, Fazekas, Riley and Cooper, 1967; Cooper, 1968; Cooper, Samara and Winsiewska, 1971).

iii) Latent phase of injury

Although cryosurgery has been designed for local discrete destruction of diseased or unwanted tissues, there is some evidence to suggest a degree of immunologic response in the patients after cryotherapy. This has stimulated a new independant field of investigation into the immunological consequences of this type of tissue destruction. This field of study has been named cryoimmunology. Early studies showed possible immunological antibody responses following cryosurgery in rabbit sex glands (Yantorno, Shulman, Gonder and Soanes, 1966; Shulman, 1967, 1968; Ablin, Witebsky, Jeiodinski and Soanes, 1971; Stoll, Barnes and Ansell, 1974).

The formation of antigenic factors during cryosurgery could be due to the modification of the molecular structure of the tissues because of the freezing. The modified new molecular structure could then be recognised by antibody forming cells (Gondor, Soanes and Vernon, 1967; Shulman, 1968; Ablin, Bronson and Soanes, 1970). Alternatively, Shulman (1968) and Ablin (1974) concluded that the antigens may be produced when the results of massive cell destruction are distributed to the surrounding areas.

Investigations in immunological reactions from tissues

other than male accessory genital organs (prostates) have been recorded, for example, liver and spleen (Helpap, Grouls, Yamashita and Breining, 1976; Ablin, 1976) both of which are parenchymal tissues.

Clinically, expected cryo-immunological response has encouraged the use of cryotherapy for tumours such as squamous cell carcinomas (Guinan et al., 1973; Guinan, Kahan, Bush and Ablin, 1974). Recent observations suggested that the immunological responses following clinical cryosurgery in man still remain less worthy than those obtained experimentally and it has been suggested that the persistance of tumours in the presence of an immunological threshold, was related both to the quality of specific tumour cell-associated antigens, and also to the specific properties of the host (Ablin, 1974, 1975; Coggin, Abrose, Dierlam and Anderson, 1974; Ablin, Guinan, Bruns, Sadoughi and Bush, 1975; Pivert, Petit, Fraisse, Cuilleret and Brizard, 1976).

E Some physical factors affecting the degree of tissue destruction

The destruction of a target tissue during cryosurgery depends mainly on the degree of cell destruction. During cold applications, the destruction of cells is related to many factors, and it must be emphasised that cells may survive under certain circumstances even if they are frozen to a very low temperature if some of these factors are missing.

The chief factors are; the temperature achieved, the size of the cryosurgical lesion or "ice-ball" and the

freeze-thaw technique employed.

The size of the "ice-ball" and the speed of its growth are directly related to the cryo-probe size and the temperature achieved and maintained at the heat exchange surfaces. The lower the temperature achieved in the cryo-probe, the lower will be the temperature in the target tissue. The diameter of the cryo-probe will also play an important role in the dimensions of the "ice-ball" (Gill, DaCosta and Fraser, 1970).

During the first fifteen minutes of a freezing episode, the "ice-ball" will increase in size to reach 80 - 90 per cent of its maximum diameter, the growth will then slow down and the ice-ball will reach its maximum dimension within 2 hours (Gill, DaCosta and Fraser, 1970). The total size of the "ice-ball" will increase and greater cell destruction will occur in successive freeze-thaw cycles. The ultimate increase in diameter of the "ice-ball" is usually achieved after five to seven repeat freeze-thaw cycles, the growth being proportionately less at each episode (Gill, Fraser and Carter, 1968). The growth of the "ice-ball" during cryosurgery is influenced by other factors such as, the area of bond between the cryoprobe and the tissue; the degree of adhesion permitting greater heat exchange; the ambient temperature, the vascularity and density of the target tissue. It has also been suggested that the duration of freezing is important in inducing cell injury and tissue destruction (Marchetta, Kumoa, Pool and Hayes, 1968; Passy, Dalaing, Turnbull and Leden, 1971).

Cooper (1965) suggested that the temperature of -22°C must be achieved to induce cellular destruction but Fraser and Gill (1967) concluded that the temperature of the tissues must only fall to below -20°C to obtain cellular destruction. Mazur (1968) concluded that at temperatures of -10°C and above, the survival of cells became increasingly dependant on the speed of the freezing procedure. Stone, Zacarian and Diperi (1969) suggested that cells will be increasingly damaged between 0°C to -40°C and Passy et al (1971) found that temperatures of -100°C to -180°C for five minutes will not necessarily produce a large cryolesion and Smith and Fraser (1974) reported that cells may be damaged at a temperature of -15°C. Mazur (1968, 1976); Mazur and Schmidt (1968) and Mazur (1976) concluded that the procedure of repeat freeze-thaw techniques is one of the important factors in inducing lethal injury to cells, while Leopard and Poswillow (1974) recommended applying pressure with the freezing to increase cell injury.

F Advantages and disadvantages of cryosurgery

Cryosurgery as a therapeutic device offers many advantages over conventional surgery in the treatment of different types of lesions. In man there is minimal scarring with good tissue remodelling (Cahan, 1965; Hansen, 1971; Torre, 1971; Chapin and Burke, 1973; Goldstein, 1977).

Tissue manipulation and controlled growth of the "ice-ball" while ensuring adequate freezing of the diseased tissues avoided excessive tissue destruction of surrounding

or underlying healthy tissue and produced a sharp line of demarcation between the necrotic tissues and the healthy tissue (Fraser and Gill, 1967; Lane, 1974). Severe haemorrhage during and after cryotherapy is avoided (Cooper and Lee, 1961; Pearson, 1968; Smith and Lipson, 1969; Lane and Burch, 1975; Smith and Fraser, 1974). Due to the direct effect of cold applications on nerve endings, cryosurgery is a relatively painless procedure (Hankel, 1969; Holden, 1972) and will give relief of pre-operative pain following treatment (Holden, 1972). Selective destruction of diseased cells leaving the architecture of the bone intact was an important factor in treating bone malignancies (Emmings, Neiders, Green, Keopf and Gage, 1966; Gage, Green, Neiders and Emmings, 1966; Marcove and Miller, 1969; Greinen, Liska and Withrow, 1975). Another advantage of cryosurgery is that it can be repeated many times without encouraging detrimental side effects such as those found in radiation therapy. The cryoprobe can be applied to sites not readily accessible to other surgical instruments (Fraser, 1975). It has been postulated that cryosurgery had an immunological reaction in the destruction of metastases of malignent neoplasms (Drylie, Jordan and Robbins, 1968; Ablin, Drylie, Jordan and Robbins, 1968). In veterinary practice particularly, cryosurgery does not necessitate sophisticated operating suites and other facilities associated with aseptic surgery; it is even suggested that cryoprobes are self sterilising.

There are a few disadvantages in using cryosurgery in veterinary practice. When treating massive tumours, many

cells escape freezing when they are located near large blood vessels and recurrence of the tumour will ensue. Clinically, the massive necrotic masses and prolonged granulation healing in pet animals are unsightly and upsetting to owners. Tissue swelling and erythematous inflammation after treatment, especially in the treatment of lesions in the oral cavity in brachycephalic animals may lead to respiratory or other problems.

Cryosurgery may produce a change in the colouration of the hair and may not be considered to be cosmetic (Lane, 1974; Thomas, Liska and Withrow, 1975).

CHAPTER 2

LIGHT MICROSCOPIC OBSERVATIONS ON NORMAL MUSCLE TISSUE AFTER CRYOSURGERY.

Introduction

Degenerative and regenerative processes in muscle tissue have been the subject of intensive clinical and laboratory investigations over the past century. These studies concentrated on different aspects and properties of change in various pathological conditions. The first important investigation into pathological change in muscle tissue was published by Zenker in 1864 after an outbreak of epidemic, typhoid fever.

The effect of cold injury on striated muscle was first studied by Volkmann in 1893, who investigated human muscle tissue after frostbite. Volkmann (1893) observed that the changes in human muscle after frostbite were the same as the changes induced by experimental freezing in Guinea Pigs.

With advances in technology, the methods of applying cold temperatures to muscle tissue have changed dramatically. The early workers used solid carbon dioxide application (Seznt, 1951; Carl, 1962) but now liquid nitrogen is the most common agent. Liquid nitrogen was first used to cool brass discs (Price et al., 1964a; Menz, 1971) with subsequent direct application. The most recent cryosurgical instruments allow a constant and continuous measured flow of cryogenic gas and fast freezing to low temperature. The use of temperature monitors (thermocouples) enables the cryolesion to be well defined. The earlier work in freezing muscle

tissue (Price et al., 1964b; Menz, 1971; Yanko et al., 1974) left many questions unanswered and the purpose of the next two chapters is to report studies on the degenerative changes caused by using modern cryosurgical techniques and the ability of the muscle to regenerate after such treatment.

Materials and Methods

i) Surgical procedures

Eight adult Guinea Pigs with an average weight of 850 gm were used in the experiment. Following general anaesthesia with fluothane, nitrous oxide and oxygen, the site of operation on the lateral aspect of the hind limb was prepared. An incision, 3 cm long was made into the area of the biceps femoris. After muscle exposure, four thermocouple needles were inserted in a circular arrangement 1 cm in diameter, followed by a 3 mm cryoprobe (CE-8 Cryosurgical system) which was applied directly over the muscle. After 3 minutes the monitoring thermocouples indicated a temperature of $-30^{\circ}C$ and the ice ball was approximately 1 cm in width and 0·5 cm in depth. After the cryogenic treatment ceased, the cryoprobe released and the muscle tissue was allowed to thaw. The skin was sutured by simple, interrupted stitches using nylon thread.

Twenty minutes, 2 hours, 24 hours, 1 week, 2 weeks, 3 weeks and 4 weeks after treatment the animals were anaesthetised as described previously and immediately small pieces of tissue were excised from the treated area. The animals were then sacrificed with an over-dose of

Expiral (0·5 cc I.C.). The pieces of tissue were approximately 1 cubic centimeter in size. The tissue block was cut into two, one half was processed for light microscopy and the other for electron microscopy.

ii) Light microscopy

The muscle biopsies were placed on a piece of card to help maintain the original shape without causing excessive contraction. The tissue was fixed in 10 per cent formal saline for 2 days at 4°C. Formal saline was used in this work because it allowed a wide variety of staining methods to be applied (Drury and Wallington, 1967).

The tissue was dehydrated and double embedded using Petofi's double embedding method (Drury et al., 1967). Wax embedded sections were mounted on wooden blocks and 30-40 sections 5-8 μm thick, of each face were cut in a rotary microtome. The strips of sections were gently applied to a glass slide and treated with 30 per cent alcohol, for 0·5 minutes, to improve their flattening properties. The sections were then lowered into a water bath for 5 minutes. Unbroken sections were selected and mounted on glass slides treated with albumin. The sections were dried at 35°C for 12 hours.

As muscle tissue contains many components, three types of stains were used. Haematoxylin and Eosin (Drury et al.,1967) was used to observe the cells, e.g. fibroblasts, endothelial cells and nuclear components of muscle tissue. Phosphotungstic acid haematoxylin (PTAH) was used to emphasise muscle striations, sarcolemma nuclei and connective tissue

(Mallory, 1900; Bulmer, 1962).

Martius scarlet blue (MSB) was used to demonstrate the structure of connective tissue and the distribution of fibrin (Drury, et al., 1962). Sections were examined in a Nikon S-Kt photomicroscope.

iii) Plastic embedding

The half of the biopsy prepared for plastic embedding and electron microscopy was cut into smaller pieces no more than 1 mm thick in one dimension. The tissue was fixed in 4 per cent glutaraldehyde in cacodylate buffer at pH 7·3 for 45 minutes at 4°C (Sabatini et al., 1963; Habeeb et al., 1968). The tissue was post fixed in 1 per cent osmium tetroxide in cacodylate buffer pH 7·3 for 45 minutes at room temperature. The blocks were dehydrated in acetone using a rapid schedule (Hayat, 1970) and then embedded in epon. Sections approximately 0·5 μm thick, were stained with toluidine blue (Richardson, 1969) and observed in the light microscope.

Results

A Normal Muscle

The biceps femoris muscle tissue was surrounded by a connective tissue layer called the epimysium (fig. 2.1). This layer consisted of thick bundles of collagen interspersed with many fibroblasts (fig. 2.2). The fibroblasts were either spindle shaped, with central basophilic nuclei and surrounded by slightly basophilic granular cytoplasm, or stellate with fine cytoplasmic extensions (fig. 2.2). From the epimysial connective tissue, many connective tissue

septa penetrated deep into the muscle tissue producing the fasciculous system (fig. 2.2, 2.3). These septa consisted of thin arrays of collagen fibrils, in between which there were many fibroblasts and a few mast cells (fig. 2.2, 2.3), these septa are called the perimysia (fig. 2.3).

Blood vessels of various sizes were found between the bundles of collagen fibres of the perimysial layer (fig. 2.3, 2.4). The structure of these blood vessels depended on the size, the capillaries consisted of a thin layer of endothelial cells, which were surrounded by collagen bundles and a few fibroblasts (fig. 2.3, 2.4). The wall of the arteries had three layers, a tunica intima which is composed of an inner-most lining of endothelial cells and a tunica media, composed of alternating layers of concentric elastic fibres and fine smooth muscle fibres (fig. 2.3, 2.4). The lumen of these cells contained red blood cells and a few leucocytes. The veins also differed in size, some of these were large, their walls consisting of tunica intima, a thin tunica media and outer-most layer, tunica adventitia (fig. 2.4). From the perimysial layer fine reticular collagen fibres surrounded each muscle fibre, this fine collagen layer was directly applied to the basal membrane of the muscle cell and it was called the endomysium (fig. 2.3, 2.5).

Within these fascicules there were many closely packed muscle cells, these were brightly stained, eosinophilic cells, and were elongated and cylindrical (fig. 2.2, 2.3). The length of the muscle cells varied from 0·3 to 5 mm,

the diameter of the cells ranged from 0·25 to 0·5 mm. The muscle cells were orientated with their long axes parallel to each other, each muscle cell was enclosed by thin smooth membrane called the sarcolemmal membrane (fig. 2.5).

Each muscle cell possessed many nuclei some of which were small and oval and others were spindle shaped, these nuclei were slightly basophilic, most of the nuclei were situated directly underneath the sarcolemmal membrane, a few nuclei were seen in different sites in the cell, mainly near the central region and these nuclei were oval and contained fine basophilic granules (fig. 2.5, 2.6).

The muscle cells contained many fine parallel thread like structures which are called the myofibrils (fig. 2.5). In sections stained with PTAH, these myofibrils had an alternating dark A zone and light I bands which were arranged in register with adjoining myofibrils (fig. 2.7). The light I bands were bisected by thin dense areas called Z lines, whereas the centre of the dark A zones were bisected by a less dense area called the H band.

Outside the muscle cells and within the endomysial collagen fibres, there were a few elongated cells - the endomysial fibroblasts.

B Twenty minutes after cryosurgery

In the light microscope and with the use of different stains, the cryolesion could be divided into three recognisable areas, each of these areas showed different

pathological changes; they can be divided as follows:-

i) Central zone: Which was the area directly under the cryoprobe and represented the centre of the lesion; the pathological changes were very severe and obvious (fig. 2.8, 2.9).

ii) Transitional zone: This region contained a mixture of severely degenerated muscle fibres mixed with partially damaged muscle fibres and a few fibres showing normal structure within their fascicules (fig. 3.10).

iii) Peripheral zone: The pathological changes were slight and the freezing episode had had only a mild effect.

i) Central zone

Muscle fibres in the central zone were separated by wide intercellular spaces (fig. 2.8, 2.9). The intercellular spaces were filled with oedematous fluid which contained degenerated cellular fragments, collagen fibrils and many degenerated fibroblasts. Within the oedematous fluid there were many rounded undifferentiated cells, which were undamaged (fig. 2.11).

The muscle fascicula arrangements were slightly disrupted and some muscle fibres were ruptured and sarcoplasm had escaped and both the endomysial layers and the sarcolemmal membranes were disrupted (fig. 2.9, 2.11). The sarcolemmal nuclei were pyknotic and disorganised, some nuclei were found outside the cell surrounded by degenerated muscle fragments and a mixture of oedematous fluid (fig. 2.11).

Severe contraction in some muscle fibres resulted

in the formation of thick homogeneous transverse bands which had an I, X, V or Y configuration (fig. 2.9, 2.11). Between these contracted transverse bands, the sparse sarcoplasm (fig. 29) contained a few thin longitudinal fibrils, some of which were connected to the transverse bands (fig. 2.12). The transverse bands had different appearances, dependent on the staining method employed (fig. 2.9, 2.12, 2.13). In some muscle fibres the contraction had caused muscle fibres to fracture (fig. 2.13), the fractures had occurred in the region between the homogeneous bands (fig. 2.14). The regions of basement membrane lying near the contracted bands had been pushed outwards, in some areas the basement membrane was ruptured and disorganised (fig. 2.14).

Some sarcolemmal nuclei were pyknotic and of variable shapes and position, while others were seen normal in structure and position (fig. 2.11, 2.12). The sarcolemmal nuclei found under or within the thick connected bands were compressed longitudinally, whilst those in the areas between the dark thick transverse bands were swollen with fine basophilic granular appearance (fig. 2.15). In some of the muscle fibres there were empty spaces between the debris of the degenerated myofibrils, these spaces differed in size and shape (fig. 2.14, 2.16).

In sections stained with MSB the epimysium showed little damage due to the freezing episode (fig. 2.17). The architecture and the orientation of the fibrous bundles of the epimysium were normal in appearance, but the adjacent

fibroblasts showed degenerative changes. The fibroblasts were irregular with undulating cell membranes, some fibroblasts contained pyknotic contracted nuclei and others swollen and irregular nuclei (fig. 2.11). The cytoplasm of the fibroblasts contained fine granules and many small fine vacules.

The perimysium was only slightly affected by the treatment. There was disorganisation and fragmentation in the continuity of the collagen bundles (fig. 2.17), associated with the separation of the fibrous bundles. Some of the collagen fibres were ruptured, disorganised and fragments were seen mixed with the oedematous fluid between the fasciculi.

The endomysium was still intact around some muscle fibres (fig. 2.18). In others it was separated from the myofibrils by clear spaces surrounding the degenerated muscle cell (fig. 2.20). In some surrounding muscle fibres the endomysium was ruptured and fragmented at points corresponding to fracture of the whole muscle fibre (fig. 2.20).

Between the severely injured muscle fibres, there were a few muscle fibres that appeared to be less injured, the normal shape had been preserved but there were no clear transverse striations, their sarcoplasm stained faintly with H & E and contained a few vacules; the sarcolemmal nuclei were contracted, irregular and pyknotic, and occupied a peripheral position (fig. 2.19).

The small blood vessels between the perimysial and

endomysial connective tissue were filled with red blood cells (fig. 2.17). The endothelial cells were slightly swollen, their nuclei were irregular and pyknotic surrounded by faintly stained vaculated cytoplasm, a few of these fine blood vessels were ruptured.

Within the epimysial connective tissue layer, medium size blood vessels showed degenerative changes; the tunica intima showed degenerate, swollen or ruptured endothelial cells, the tunica media contained irregular, disrupted, fine, smooth muscle cells which were irregularly oriented within their fibrous sheath. The tunica adventia was slightly swollen and showed distortion of the concentric pattern of the fibrous layer. Blood stagnation and engorgement was evident, some of these vessels were ruptured with consequent blood loss. The blood cells were scattered and mixed with the oedematous fluid which was distributed between the muscle fibres (fig. 2.21). In other vessels, the endothelial cell nuclei were situated at the periphery of a protrusion of cytoplasm which had extended far into the lumen of the blood vessel. These nuclei contained fine basophilic granules (fig. 2.21). Generally, the small veins contained more blood cells than the small arteries in the same area (fig. 2.17). The connective tissue elements of these vessels were still virtually intact (fig. 2.17).

In sections stained with MSB and H & E, small fine threads of fibrin could be seen mixed with the fluid which had escaped from the damaged blood vessels (fig. 2.21).

In some places the capillary walls had ruptured, in other regions the capillary wall had been compressed between swollen muscle fibres, which had resulted in closure of the lumen.

ii) Transitional zone

In the transitional zone the muscle fibres showed different degrees of injury, ranging from highly disrupted and contracted muscle fibres to a mildly affected muscle cell, some of the muscle fibres showed partial injury, in which the portion near the central zone showed more disruption than the part nearer the periphery of the transitional zone (fig. 2.22, 2.23). The muscle fibres were separated by fairly large intercellular spaces containing oedematous fluid (fig. 2.22, 2.23). There were more polymorphs present in the intercellular spaces in this zone than in the central region (fig. 2.24).

At one extreme some of the muscle fibres contained a few incomplete dark transverse bands indicating a loss of the normal transverse striations (fig. 2.22). Some swollen muscle fibres showed no clear dark transverse bands and contained widely separated myofibrils, lying parallel to the longitudinal axis of the cell (fig. 2.25). The myofibrils were embedded in homogeneous finely granular eosinophilic matrix (fig. 2.26).

The sarcolemmal nuclei were situated at the periphery of muscle fibres and were either pyknotic or slightly swollen (fig. 2.24, 2.25, 2,26). The endomysial layer was separated from the sarcolemmal membrane (fig. 2.25).

Some of the collagen fibres of the endomysial layer were distorted and separated. Towards the periphery of the transitional zone there were a few muscle fibres with normal transverse striations; their sarcolemmal nuclei were still in the normal position but were slightly swollen. A few sarcolemmal nuclei in some of the muscle fibres were found towards the centre of the cell, these nuclei were rounded and slightly swollen (fig. 2.27).

In partially damaged muscle cells, the damaged part showed the same changes as observed in the damaged muscle fibres in the central zone, other parts showed no clear pathological changes, except the transverse striations were irregular.

With MSB stain in some areas the endomysial connective tissue surrounding the muscle fibres was still intact. Even surrounding the severely affected muscle cells, the endomysial layer was still intact or occasionally was a little distorted. This distortion was associated with focal separation of the collagen fibres.

Some of the fibroblasts between the muscle cells showed early symptoms of degeneration but most of the fibroblasts were still intact. The blood vessels were still intact (fig. 2.28) but were congested and filled with blood cells. Some of these blood vessels showed swollen endothelial cells. Small blood vessels, mainly capillaries at the margins of this zone nearest the central zone showed the same degenerative changes as those seen in the central zone (fig. 2.29). Scattered between these blood vessels

(fig. 2.29) were many muscle fibres which were intact and well preserved.

iii) Peripheral zone

Most muscle fibres in this region showed little damage. In general, the muscle cells had well preserved transverse striations. The sarcolemmal nuclei were normal and situated in the normal peripheral position (fig. 2.30). However, a few muscle fibres showed early stages of hyaline degeneration (fig. 2.31). Other cells were slightly swollen (fig. 2.30) and their transverse striations were irregular. The endomysium was well preserved, some of the fibroblasts were swollen, oval in shape and contained granular cytoplasm and large central basophilic nuclei. The blood vessels were intact, congested with red blood cells and a few leucocytes.

C Two hours after treatment

In the specimens taken two hours after treatment, the degenerative changes in the muscle fibres were severe. The three zones were much more clearly defined than in the specimens taken 20 minutes post treatment.

i) Central zone

There were several noticeable changes between tissues taken 20 minutes and two hours post treatment. In the central zone the muscle fibres showed severe injury (fig. 2.32), after two hours, the irregular dense transverse bands were the only distinguishable remnants of the injured muscle fibres. Instead of the irregular I, X and Y shaped transverse

bands seen 20 minutes post treatment, interspersed with fine granular material, the muscle fibres consisted of swollen, dense, hyaline masses surrounded by empty spaces (fig. 2.32, 2.33).

Accompanying these changes in the appearance of the dense bands, the staining properties of the muscle had also changed (fig. 2.33), e.g. with MSB the muscle cells which normally stained red were yellowy orange. The transverse bands stained with H and E were deep pink 20 minutes after treatment but the dense masses were pale pink in tissue after 2 hours (fig. 2.32). The dense masses in sections stained with PTAH had changed from dark blue interspersed with small pale blue areas at 20 minutes to show a pale blue central region with a dark blue peripheral band after two hours. The region between the hyaline masses showed no staining reaction in two hour specimens.

The sarcolemmal nuclei two hours after treatment showed various reactions, some remained in their peripheral position adjacent to the basement membrane (fig. 2.32, 2.33), but were pyknotic and flattened. Others had moved to other regions of the muscle fibre and these were swollen and contained basophilic granules, several sarcolemmal nuclei were ruptured and nuclear debris was seen scattered and mixed with necrotic sarcoplasmic material (fig. 2.34). Some nuclei were seen in the intercellular spaces mixed with necrotic muscle cell debris, frequently ruptured and distorted (fig. 2.32, 2.33). A few muscle fibres in the central zone were swollen and showed typical "Zenkers

degeneration", these cells contained swollen distorted nuclei scattered in homogeneous sarcoplasm, the sarcoplasm was stained pale pink with H & E stain and contained a few small vacules. Even in the central zone there were a few muscle fibres still exhibiting relatively less damage (fig. 2.34). The oedematous fluid between the muscle tissue components had increased.

There was an increase in the distortion of the collagen fibres and connective tissue generally after two hours. In sections stained with MSB, the epimysium was still seen surrounding the muscle fascicules, this layer showed more distortion than that observed within twenty minutes post treatment, the perimysial layer reacted similarly (fig. 2.36). The endomysial layer was still well preserved, even though the muscle fibres were severely damaged (fig. 2.37). The endomysial walls had retained their elliptical shape (fig. 2.37) but a few endomysial tubes were disrupted and fractured (fig. 2.39). With H & E the connective tissue fibroblasts were swollen, oval or rounded and contained acidophilic granular cytoplasm, their nuclei were degenerated, pyknotic or swollen. Some fibroblasts were found in the large intracellular spaces away from the normal position juxtaposed to the muscle fibres (fig. 2.38), these fibroblasts were still attached to fine thin fibrils of the endomysium (fig. 2.39a).

The blood vessels in the region were affected in many ways (fig. 2.39a). Large blood vessels of the epimysium showed more distortion than was seen twenty minutes after

treatment, their lumen contained fine microthrombi and many red blood cells as well as a few leucocytes. Other smaller blood vessels showed extensive injury, in which the vessel walls were ruptured, the blood had escaped and mixed with the oedematous fluid.

The venules and capillaries were severely injured and distorted, especially the endothelial cells, which were either highly swollen with irregular nuclei or were ruptured (fig. 2.40). The lumena of the capillaries were reduced in diameter and contained red blood cells and leucocytes. Within the blood vessels of this region the leucocytes were mainly eosinophils, monocytes and neutrophils, most of the polymorphs had adhered to the walls of the damaged vessels (fig. 2.35).

The muscle cells were separated by large intercellular spaces which contained profuse oedematous fluid, polymorphs, necrotic cellular debris, degenerated fragments of nuclei elements, collagen fibres, undifferentiated rounded cells (fig. 2.41).

ii) Transitional zone

This area represents the intermediate zone between the severely degenerated muscle fibres and the peripheral muscle cells which were less affected (fig. 2.36). The thickness of the transitional region varied from 10 - 15 muscle cells.

The changes in this region showed a gradation from severely degenerated necrotic muscle cells to others which were only very mildly affected (fig. 2.42, 2.43, 2.44).

The cells nearest the central zone were the most heavily distorted and necrotic, and passing towards the periphery of the zone the damage was less.

The severely injured muscle fibres in this zone had the same appearance as those seen in the central zone (fig. 2.42, 2.44). Some cells showed "Zenkers degeneration" whilst others had clear longitudinal myofibrillar arrangement, these myofibrils were separated by clear spaces, in some of these cells the transverse striations were still existing but were irregular (fig. 2.45). Some of the muscle fibres were partially injured, and the injured region of the cells showed the same features as the severely damaged muscle cells of the central zone. The undamaged region of these cells showed irregular transverse striations. Near the periphery of the zone there were cells with no obvious degenerative changes, but were slightly swollen, some of their sarcolemmal nuclei were seen rounded and granular (fig. 2.44).

In sections stained with PTAH, some muscle fibres were stained pale blue, others were well stained and deep blue (fig. 2.45), the density of the staining properties was reflected in the degree of injury to the muscle fibres. The pale blue cells were the most severely injured muscle fibres. The overall impression was an increase in blue colouration from the central zone to the periphery of the transitional zone.

In sections stained with MSB, the fascicular structure of the muscle bundles was generally well preserved. The

epimysial, perimysial and endomysial connective tissue bundles and fibrils were almost intact, but near the severely damaged muscle fibres, the perimysial and especially the endomysial connective tissue showed partial distortion.

Most of the blood vessels of this zone were still intact and unchanged, a few showed degenerative changes in their endothelial cells, especially in those near the central zone. The connective tissue bundles surrounding these vessels were filled or engorged with oedematous fluid, red blood cells and large numbers of polymorphs (fig. 2.44).

The oedematous fluid in this zone showed an increase in quantity from 20 minutes to two hours post treatment. Within the oedema there was an increase in number of polymorphonuclear leucocytes as well as many rounded intact cells (fig. 2.44).

iii) Peripheral zone

The muscle cells of this zone were still well preserved and almost intact, however, some of the cells were slightly swollen and had the appearance of a syncytium as the intercellular spaces were very narrowed (fig. 2.46). At times it was difficult to recognise the endomysial layer and the cellular membrane, as the collagen fibrils were compressed (fig. 2.46). In most instances, the transverse striations of the muscle fibres were still intact with distinct A, I bands, the sarcolemmal nuclei were still intact, although a few were pyknotic and irregular. A few muscle cells had lost the normal transverse striations

and the myofibrils showed longitudinal separation. The epimysial connective tissue layer was still intact, with intact fibroblasts, a few polymorphs were seen between the connective tissue bundles; the perimysium was also intact. Large blood vessels between the epimysial layer were still intact, congested with red blood cells and many leucocytes. It was difficult to recognise the blood capillaries between the swollen muscle fibres as they had been compressed.

D Twenty four hours after treatment.

i) Central zone

The majority of the muscle fibres in this zone were severely necrotic, the normal shape and structure of the muscle fibres had been totally distorted (fig. 2.47, 2.48).

The severely damaged muscle fibres contained irregular necrotic hyaline masses, surrounded by polymorphs (fig. 2.48). In other muscle fibres, in the same region, the thick transverse bands first seen twenty minutes post treatment were very swollen and surrounded by degenerate, irregular profiles of myofibrils (fig. 2.49). A few endomysial tubes were virtually empty but many polymorphs and histocytes had infiltrated through the endomysium to occupy the lumen of these tubes (fig. 2.47, 2.49). The sarcolemmal nuclei of the degenerated muscle cells were shrunk, pyknotic and a few were highly disorganised (fig. 2.47, 2.48, 2.49). Some of the nuclear fragments of the ruptured sarcolemmal nuclei were seen mixed with the degenerated muscle debris.

In some areas within the central zone, the muscle fibres showed complete autolytic changes, the matrix consisted of faintly stained masses, surrounded by many macrophages.

In the same region, stages in the progressive hyalin degeneration could be seen, cells were swollen and the sarcoplasm was homogeneous and stained pale pink with H and E (fig. 2.47), with PTAH these muscle cells showed no transverse striations, the sarcolemmal nuclei were degenerate and pyknotic. Some of these degenerate nuclei had moved to occupy different areas of the muscle fibre.

The endomysial collagen fibres showed a high degree of resistance as even around the severely degenerate muscle fibres, most of the endomysial collagen fibrils were still intact (fig. 2.47). There was some focal separation of the collagen fibres around some degenerate muscle cells. Endomysial fibroblasts were degenerate and with granular vaculated cytoplasm and pyknotic nuclei.

The intercellular spaces between the muscle fibres in the central zone were still occupied by oedematous fluid which contained a large number of polymorphonuclear leucocytes, erythrocytes, macrophages and undifferentiated large rounded cells with slightly basophilic cytoplasm and large centrally positioned rounded nuclei. Fine reticular threads of fibrin, small amounts of necrotic muscle cell debris and fragments of collagen bundles were also present.

The leucocytes and macrophages were heavily concentrated around the highly degenerate and necrotic muscle cell (fig.2.51a).

The blood vessels within this zone showed many changes, especially in the cellular part. In general, the connective tissue layers of the blood vessels had mostly retained their normal shape, in a few blood vessels the collagen bundles were still separated or partially fractured, the endothelial cells were either degenerated and detached from the lumen or were disorganised, swollen, with acidophilic cytoplasm and irregular pyknotic nuclei. The lumena of these vessels were filled with red blood cells and numerous leucocytes. Some of these leucocytes had adhered to the vessel wall and others had migrated through the vessel wall and were mixed with the oedematous fluid. Small blood vessels, venules, arterioles and blood capillaries showed similar endothelial cell changes and a few were ruptured and disorganised.

ii) Transitional zone

Some of the muscle fibres in this zone showed hyalin degeneration (fig. 2.49), others were swollen and contained homogeneous, granular matrix, and a few muscle fibres showed longitudinally separated myofibrils (fig. 2.50).

There were a few muscles showing partial injury, the intact portion was swollen with separated myofibrils, and the degenerate portion of the cell was seen as fragmented irregular masses, surrounded by polymorphs and macrophages. In the injured muscle fibres, the sarcolemmal nuclei were pyknotic and had moved from their normal peripheral position. Other sarcolemmal nuclei were swollen, rounded and contained irregular slightly basophilic nucleoli (fig. 2.49, 2.50).

Some muscle fibres at the periphery of this zone showed nearly normal transverse striations with clear A, I bands and H, Z lines (fig. 2.50).

There was less oedema in this zone than in earlier biopsies, the oedematous fluid contained red blood cells, polymorphs, macrophages, intact and degenerate fibroblasts, many rounded with large rounded nuclei and also irregular masses of degenerate tissue (fig. 2.51). The polymorphs and macrophages in this zone were again concentrated near the damaged muscle fibres and the injured portions of the partially damaged muscle cells (fig. 2.50, 2.51). The epimysium, perimysium and endomysium were still intact. The majority of the blood vessels were well preserved but they were congested with red blood cells and leucocytes (fig. 2.52).

iii) Peripheral zone

The general histological picture of the muscle tissue showed this zone still intact. The transverse striations of the muscle fibres were present, although some of the muscle fibres were slightly swollen, a few muscle cells showed irregular, disrupted transverse striations. The sarcolemmal nuclei were swollen and rounded with prominent basophilic nuclei, these nuclei had migrated towards the central axis of the cell to form a chain of rounded or oval nuclei. The endomysium was still intact surrounding the muscle fibres. The fibroblasts were still intact spindle in shape. Between the connective tissue bundles there were a few polymorphs and macrophages. The intercellular

spaces between the muscle cells were reduced and narrowed. The blood vessels in this area were still intact, the lumena were dilated and contained red blood cells and many leucocytes.

E One week after treatment

The most significant observation in the lesion one week after treatment was evidence of muscle regeneration. Tissue damage was still much in evidence along with signs of active removal of the damaged cell fragments.

i) Central zone

In the very middle of the lesion the scattered muscle fibres were separated by newly-formed scar tissue (fig. 2.53) consisting of collagen fibres, fibroblasts and small blood vessels. There appeared to have been infiltration of active fibroblasts into the area with a consequent increase in collagen bundles. Macrophages and leucocytes surrounded the degenerate muscle fibres (fig. 2.53). Peripheral to this region the muscle fibres were more numerous (fig. 2.54) and were surrounded by large numbers of leucocytes and macrophages. Several leucocytes and macrophages had penetrated the endomysial tubes and appeared to be involved with phagocytosis of the homogeneous cell remnants (fig. 2.55). The sarcolemmal nuclei were irregular and pyknotic and some were fractured and degenerate (fig. 2.55). These nuclei were irregularly scattered throughout the muscle fibre. The endomysial layers of the damaged muscle fibres were largely intact (fig. 2.56).

In sections stained with MSB, the connective tissue

between the muscle fibres was seen as irregular accumulations between the damaged muscle fibres (fig. 2.57).

In the area surrounding the central core, the intercellular spaces contained fewer macrophages and polymorphs but there was an increase in the spindle cells, stellate cells and multi-nucleated cells (fig. 2.58). There were many bundles of fibrin-like material in the intercellular spaces (fig. 2.59).

Peripheral to the collagen bundles and scattered muscle fibres of the central zone (fig. 2.59), the first signs of muscle fibre regeneration were seen (fig. 2.60). The regenerating muscle fibres of the central zone were first seen as oval or spindle-shaped structures containing amorphous cytoplasm and one or more basophilic rounded nuclei (fig. 2.60). The long axes of the regenerated myotubes conformed to the original fascicular muscle fibre arrangement (fig. 2.60, 2.61). Often there were regenerated myotubes present in one fascicule but not in the neighbouring one (fig. 2.62). The regenerated myotubes were surrounded by connective tissue (fig. 2.60, 2.61, 2.62) containing fibroblasts and collagen bundles.

Blood vessels of all sizes had a normal appearance and the lumina of the vessels were packed with leucocytes and red blood cells (fig. 2.61).

ii) Transitional zone

In this zone there were three types of degenerate muscle fibres as well as evidence of active regeneration. The three types of degenerate muscle fibres were as follows:-

The first type showed very reduced endomysial tubes containing irregular clumps of debris, surrounded by many macrophages and leucocytes. The second type of muscle fibres were almost intact, but were swollen with irregular transverse striations and centrally positioned sarcolemmal nuclei. The rounded basophilic sarcolemmal nuclei were arranged in long chains and contained many basophilic nucleoli. The third type of muscle fibres were partially damaged, with the damaged regions nearer the central zone. The damage was seen as either empty, clear areas, or as clusters of cell debris surrounded by macrophages (fig. 2.63).

The connective tissue components were the same as those in the central region.

Regenerated sarcoblasts or myoblasts which contained several oval nuclei and faintly basophilic cytoplasm were also found near the junction of the transitional and central zones (fig. 2.63).

iii) Peripheral zone

Mostly, the muscle fibres in this zone were intact with clear transverse striations. Between the intact muscle cells there were a few cells containing irregular and incomplete transverse striations. The sarcolemmal nuclei usually retained their normal position but were larger and more rounded (fig. 2.64).

F Two weeks after cryosurgery

i) Central zone

The centre most part of the lesion contained only a few scattered, necrotic muscle fibres, debris and phagocytes (fig. 2.65). The major constituent of the centre of the lesion was connective tissue (fig. 2.65) containing many active fibroblasts (fig. 2.66). Between the collagen bundles there were many oval or polyhedral plasma cells with rounded granular nuclei (fig. 2.66).

The central zone contained numerous small blood vessels which consisted of an endothelial layer surrounded by collagen bundles (fig. 2.65). The lumena of these vessels contained many red blood cells and a few leucocytes.

Towards the periphery of the central zone there were several single, regenerated, multi-nucleate myotubes (fig. 2.67) scattered between the collagen bundles. The myotubes were at different stages of development. The newly regenerated muscle fibres were found juxtaposed to degenerate muscle fibres (fig. 2.68). The regenerated muscle fibres showed sarcoplasm of denser staining containing round nuclei. Whereas the degenerate muscle fibres contained irregular and pyknotic nuclei and the sarcoplasm was stained very faintly (fig. 2.68). The myotubes showed different stages of development with the more mature myotubes demonstrating fine transverse striations. With maturity the nuclei in the regenerated myotubes assumed the normal flattened outline.

At the periphery of the central zone the connective tissue component decreased and the numbers of muscle fibres

increased (fig. 2.69). The regenerated muscle fibres were thin and long.

Some of the regenerated muscle fibres indicated secondary degeneration and were surrounded by macrophages and polymorphs (fig. 2.70).

ii) Transitional zone

In a section stained with MSB the muscle fibres showed continuous regeneration with growth towards the central zone (fig. 2.71). The continuously regenerated muscle fibres were identified by the larger numbers of rounded basophilic nuclei (fig. 2.72) present in the bud which had developed towards the central zone. The regeneration had commenced in the undamaged endomysial tube in the region of intact myofibrils (fig. 2.72). The partially damaged muscle fibres were able to regenerate continuously and the newly formed part of the muscle fibre appeared to advance towards the central zone.

At high power (fig. 2.73) the tip of the bud, which had been the part of the muscle fibre most affected by the treatment, contained newly formed myofibrils and nuclei. The intact endomysial tube was almost filled with newly regenerated sarcoplasmic material (fig. 2.73). The regenerated muscle fibres were surrounded by collagen and fibroblasts (fig. 2.72, 2.73). The regenerated region of a muscle fibre was distinguishable from the undamaged portion by its scalloped outline (fig. 2.74).

iii) Peripheral zone

The peripheral zone showed a slight increase in the

numbers of collagen bundles but was otherwise normal.

G Four weeks after cryosurgery

Sections taken from biopsy specimens four weeks post treatment showed several progressive, regenerative changes. The chief differences were seen in the transitional zone where the partially injured muscle fibres had fully regenerated. The regenerated muscle fibres had penetrated deep into the central zone. The three zones were much less distinguishable and the lesion will be described as two zones, the central zone and the surrounding peripheral zone.

i) Central zone

The major part of the central zone consisted of dense collagen bundles interspersed with active fibroblasts (fig. 2.75). Within the connective tissue there were many small rounded, blood vessels (fig. 2.75). There were only a few scattered remnants of muscle cells and these were surrounded by collagen bundles and a small number of phagocytes. Towards the periphery of the central zone the numbers of collagen bundles had decreased but there was an increase in the numbers of fibroblasts (fig. 2.76). In this area there were also many regenerated, single, rounded muscle cells, some of which demonstrated irregular, fine myofibrils (fig. 2.77). The regenerated muscle cells contained many rounded basophilic nuclei which were situated centrally.

ii) Peripheral zone

The process of regeneration and repair of the partially injured muscle fibres seen in section E was completed by twenty eight days post treatment. The regenerated regions of the cells had a wavy or spiral appearance and contained many nuclei (fig. 2.78). The regeneration was directed towards the centre of the lesion (fig. 2.78) and the more central regions of the cells were surrounded by collagen bundles (fig. 2.79).

The perimysial connective tissue surrounding the regenerated muscle fibres was thicker than normal (fig. 2.80). The thickness of the perimysium decreased with distance from the centre of the lesion (fig. 2.81).

Towards the periphery of the biopsy the majority of the muscle fibres were intact and normal. Muscle cells were surrounded by a thin endomysium and were arranged in fascicules. The muscle fibres showed normal transverse banding and the sarcolemma nuclei occupied peripheral positions juxtaposed to the sarcolemma membrane.

Summary of Results

1. Twenty minutes after treatment. The central zone muscle cells showed the effects of severe contraction, some were completely ruptured and disorganised, others showed the formation of thick hyaline bands. Nuclei were pyknotic and irregular but connective tissue elements showed little damage. The blood vessels were severely injured.
2. Two hours after treatment. The central transitional

and peripheral zones were more clearly defined. In the central zone the swollen, irregular muscle cells showed thick transverse bands. The collapsed endomysium was the only remnant in some cases. In the transitional zone, the endomysial tubes of all the cells were preserved although the contents suffered varying degrees of injury. The number of leucocytes and the oedema had increased.

3. Twenty four hours after treatment. The large increase in leucocytes and macrophages indicated that the necrotic debris was being actively removed. The amount of oedema had reduced. Fibrin had been excreted. Fibroblasts accompanied by fusiform mononuclear cells were found in the centre of the lesion.

4. One week after treatment. The first signs of muscle fibre regeneration appeared seven days after treatment. Scar tissue was present in the central zone. The oval regenerated muscle fibres contained amorphous cytoplasm and several basophilic nuclei.

5. Two weeks after treatment. The periphery of the central zone was characterised by many single, scattered multi-nucleate myotubes at different stages of regeneration. Some regenerated muscle fibres were surrounded by macrophages and polymorphs and showed signs of secondary degeneration. There was an increase in the thickness and density of the connective tissue bundles associated with a decrease in the number of fibroblasts.

6. Three weeks after treatment. The lesion had the same appearance as after two weeks.

7. Four weeks after treatment. There were many regenerative changes evident in the transitional zone. The central zone consisted of scar tissue, the periphery of which was penetrated by isolated regenerated muscle fibres.

Discussion

The pathological changes in muscle tissue following cryosurgery showed both degenerative and regenerative changes during the period of study. The full extent of the injury was evident two hours after treatment. By twenty four hours post treatment, tissue repair processes were present. Muscle fibre regeneration was seen within one week after treatment.

Volkman (1893) was the first to observe severe, irreversible contraction of muscle fibres after frost bite in man and experimental freezing in the Guinea Pig. The myofibrils were disrupted at the Z line and formed irregular thick transverse bands, which was referred to as the shredded appearance (Volkman, 1893). The central zone of the experimental lesion showed the same phenomenon twenty minutes after treatment.

From his studies on ischemic muscle, Le Gros Clark (1946) concluded that the shredded appearance or discoloidal phenomenon was due to the action of the formalin fixative and was an artefact. However, Harman (1947) stated that the appearance was a result of real changes associated with necrobiosis of the muscle cells. The thick transverse bands also formed after severe Faradization during which

the muscle cells were shortened 60 per cent of their length (Nageotte, 1937). Exposure to dry ice for forty seconds also produced the same features. Pearson (1962) also reported a sharp demarcation between injured and uninjured regions after local freezing with dry ice.

Twenty four hours after treatment the degree of oedema had decreased and many active fibroblasts were seen in the central zone. Associated with the large numbers of fibroblasts were fusiform mononuclear cells which were concentrated at the junction of the central and transitional zone. The presence of large numbers of these cells in the midst of a pathological lesion in muscle tissue has led to the speculation that transformed fibroblasts are responsible for new muscle cell formation (Tello, 1922; Levander, 1945, 1955; Bergman, 1962).

However, Walker (1963) labelled connective tissue cells in muscle tissue before wounding and showed that fibroblasts do not form muscle cell precursors. The origin of new muscle fibres has been the subject of considerable debate over the last decade. One school of thought has postulated that the cellular nuclei of degenerate muscle cells are responsible for new muscle fibre production.

The other school concluded that the newly formed muscle fibres developed from the satellite cells (Carlson, 1973). The destruction of muscle fibres and nuclei observed in the centre of the lesion after cryosurgery was so great, it is thought most improbable that the nuclei of the degenerate muscle fibres could give rise to regenerated cells. The

observations supported the second idea of satellite cell origin for muscle regeneration in the most severely injured region. This type of muscle fibre regeneration has been called embryological or discontinuous regeneration (Dawson, 1909; Speidel, 1938).

The term "embryological" applied to the regeneration of muscle cells in the adult indicates the similarity between developing muscle cells and regeneration. The first evidence of myogenesis in the embryo is the elongation or spindle shaped cells, which contain no myofibrils (Holtzer, Marshal and Finch, 1957). These mononucleate cells known as myoblasts (Codlewski, 1901) show no evidence of mitotic activity.

The myoblasts fuse to form the muscle fibre which is a multinucleate myotubes syncytium (Okazaki and Holtzer, 1965). The synthesis of actin and myosin occurs after cell fusion (Coleman and Coleman, 1968). After muscle cell maturation many mononuclear cells were seen juxtaposed to the endomysium and the cell wall. These cells were called satellite cells (Muro, 1961). Holtzer (1970) postulated that these cells are resting myoblasts and one possible source of myogenic precursors during cell regeneration after injury. A closer study of the cryolesion between 24 hours and one week may cast more information on satellite cell activity during muscle fibre regeneration.

The term discontinuous regeneration was coined as a compliment to the term continuous regeneration, which is the name given to the repair of partially damaged muscle fibres. Some of the muscle fibres observed in the investigation

were only partially injured, the damaged region was nearest to the centre of the lesion. The partially injured cells retained an intact endomysial tube into which phagocytes migrated. When the necrotic sarcoplasm had been removed the endomysial tube filled with large nuclei. New sarcoplasm appeared in the damaged portion of the cell which was of normal endomysial tube appearance. The new sarcoplasm elements forming as a continuation from the existing healthy sarcoplasm. Complete regeneration of the myofibrils takes several months (Le Gros Clark, 1946; Field, 1960; Adams et al., 1962).

The events in the continuously regenerated muscle fibres were similar to those seen in regenerating muscle fibres after different types of injury such as ischemia (Le Gros Clark, 1946), simple section (Adams et al., 1962) and crushing (Field, 1960).

In the cryolesion the discontinuously regenerated muscle fibres formed in the area of greatest injury where the muscle fibres were completely destroyed. If the newly formed muscle fibres had developed from the satellite cells, these were thought to have originated from the transitional zone. The injury in the central zone was so extreme that no viable satellite cells could have survived. The continuous regeneration occurred in the transitional zone where the muscle fibre damage was only partial.

The discontinuously regenerated muscle fibre cells in the central region showed secondary degeneration in the latter stages of the study. A possible explanation for

this could well have been the lack of innervation. The nature of the regeneration of innervation to injured muscle cells is not well understood. Denervation at the myotube stage of muscle development has been shown to retard the rate of maturation (Allbrook et al., 1951). Schultz (1978) showed degeneration of regenerated muscle cells after denervation. The degenerative changes were associated with increased connective tissue formation (Betz, Firkt and Reznik, 1966). However, the evidence is inconclusive and the changes in the discontinuously regenerated muscle cells may well be due to numerous other factors, for example, nutrition.

The muscle fibres, which showed continuous regeneration, did not show secondary degeneration.

The connective tissue was the least affected of the tissues. As a result the shape of the larger blood vessels, endomysia and permysia were preserved.

The severity of injury to blood vessels has been shown to be related to the size of vessel and the duration of freezing (Whittaker, 1973). Major blood vessels have been shown to have a strong resistance to freezing to $-180^{o}C$ (Cooper et al., 1971). The endothelial cells were the most susceptible to damage caused by freezing (Taylor et al., 1950).

The injury caused by the cryosurgery was well defined and well contained.

CHAPTER 3

ULTRASTRUCTURAL OBSERVATIONS OF THE DEGENERATIVE CHANGES IN MUSCLE TISSUE AFTER CRYOSURGERY.

Introduction

The studies reported in chapter 2 showed that the major degenerative changes took place within 24 hours after cryosurgery of the normal muscle *in vivo*. By one week after treatment regeneration of the muscle tissue had commenced. As the ultrastructural observations on the effects of cryosurgery of muscle were so extensive it was decided to report the degenerative and regenerative processes in two separate chapters. This chapter is concerned with the ultrastructural observations of the degenerative changes that took place in the first 24 hours after treatment. A brief account of the ultrastructure of normal muscle tissue is included in this report.

Materials and methods

(i) Surgical procedures

As in chapter 2, materials and methods section (i).

(ii) Electron microscopy

The biopsy material was prepared for electron microscopy as described for plastic embedding in chapter 2, materials and methods, section (iii). After sections were observed in the light microscope a suitable area of the block was selected for thin sectioning. Sections 50 nm to 60 nm thick were cut and stained with uranyl acetate in 50 per cent

ethanol and Reynold's (1963) lead citrate. The sections were observed in an AEI 6B electron microscope.

Observations

A Normal muscle tissue

The muscle cells in the normal untreated biopsies from adult Guinea Pigs were elongated, cylindrical and orientated longitudinally to form the muscle mass. The ultrastructure of striated muscle cells has been well documented in recent years. The appearance of the untreated muscle cells was essentially the same as has been reported in the literature (Bloom and Fawcett, 1975; Gauthier and Padykula, 1966).

The normal muscle cells in the untreated biopsies were enclosed by a sarcolemmal membrane which consisted of a continuous electron dense amorphous basal lamina 50 nm thick (fig. 3.1, 3.2). Many extracellular collagen fibres insert into the basal lamina (fig. 3.1, 3.2). The cell membrane was separated from the intercellular membrane by a narrow lamina lucida (fig. 3.2) on the aspect of the basal lamina (fig. 3.2). In sections double stained with lead citrate the cell membrane was very electron dense (fig. 3.2).

The major constituent of the muscle cells were the myofibrils which were arranged in longitudinal bands orientated parallel to the long axis of the cell. Each myofibril was composed of numerous myofilaments which were of two types, thick and thin (fig. 3.3, 3.4). The

thick filaments have been reported to be myosin (Johanns, 1974) and the thin filaments of actin (Johanns, 1974). The arrangement of the filaments created longitudinally alternating A and I bands the former having a light H band and M line and the latter a dark Z line (fig. 3.1, 3.4).

In the A band, thick and thin filaments interdigitate but the I band has only thin filaments and the H band only thick filaments (fig. 3.5). Each thick filament is surrounded by six thin filaments (fig. 3.3). The thick and thin filaments are connected by cross bridges (Bloom and Fawcett, 1968), at the centre of the H band is a further density called the M line (fig. 3.5) which consisted of thin transverse filaments M bridges which connect each thin filament to its five neighbours. The Z line often appeared as an amorphous band at low power (fig. 3.1) but at high power a zig-zag pattern could be seen (fig. 3.5).

The myofibrils were surrounded by sarcoplasm (fig. 3.1) containing mitochondria ribosomes, glycogen particles, and sarcoplasmic reticulum (fig. 3.6, 3.7). The Golgi bodies were situated in the perinuclear sarcoplasm. The mitochondria were numerous, elongated and packed between the myofibrils (fig. 3.7). Other shorter mitochondria were seen near the sarcolemma nuclei and the poles of the cell.

The sarcoplasmic reticulum (SR) forms a lace like sleeve around the myofibrils (fig. 3.8). At the H band of the A bank the SR is continuous round the myofibril (fig. 3.8). From this continuous collar finger-like

projections run parallel to the myofibril along the A-band to the sides of the Z line where they terminate with swollen ends, the terminal cisternae (fig. 3.4, 3.8). The terminal cisternae flank the transverse process of the sarcolemma membrane (fig. 3.4, 3.8). The three vesicles are known as a triad and in cross section appear as two rounded vesicles on either side of a narrow, long, oval vesicle (fig. 3.4). The transverse process (fig. 3.8) is an invagination of sarcoplasmic reticulum and in longitudinal section (fig. 3.8) small projections from the SR cisternae were seen connected to the transverse process by amorphous material. The small projections are known as SR feet and occur in parallel rows one each side of the transverse process (fig. 3.8). In each sarcomere there are two triads (fig. 3.1).

The sarcolemma nuclei are elongated, flattened and orientated with their long axes parallel to the long axis of the cell (fig. 3.10).

The nuclei were usually situated immediately adjacent to the sarcolemma membrane (fig. 3.10). One or two nucleoli were prominent within the nucleus (fig. 3.10). The nuclear membrane was smooth and thin.

Closely associated with the muscle cells, situated between the sarcolemma membrane and the basal lamina were short fusiform cells which are known as satellite cells (fig. 3.11). The satellite cells were undifferentiated and the nucleus occupied a large proportion of the cell (fig. 3.11). There was a distinct satellite plasmalemma

separating the cell from the sarcolemma (fig. 3.11). The sparse cytoplasm of the satellite contained cell ribosomes, occasional mitochondria and a little rough E.R. (fig. 3.11).

B Twenty minutes after treatment

The light microscope observations of the cryolesion defined three zones (chapter 2). The ultrastructural studies of the cryolesion were also divided into three zones.

The zones were:-

i) Central zone - in which the ultrastructural changes in tissue were extreme.

ii) Transitional zone - in which the injury at the ultrastructural level was less severe.

iii) Peripheral zone - in which the changes were very slight.

i) Central zone

In the central zone the treatment produced severe myofibril contraction which resulted in rupture of the myofibrils (fig. 3.12). The characteristic banding of the myofibrils had been disrupted (fig. 3.12, 3.13, 3.14). The dark bands present were the remnants of the H line and of the A band (fig. 3.14). The majority of the Z-lines had been destroyed (fig. 3.13, 3.14). The distances between the H line were variable (fig. 3.13, 3.14). The rupture of the myofibrils occurred in the I band (fig. 3.13, 3.15). The fracture line of the myofibrils was irregular

(fig. 3.15) which resulted in fragments of various sizes with jagged edges (fig. 3.12). Some myofibril fragments still showed tenuous contact with the severely contracted region of the fibril (fig. 3.12). The loss of banding in some myofibrils was the result of rupture of some of the myofilaments but with no associated myofibril fracture (fig. 3.14, 3.15). The fracture of several myofibrils at the same point gave rise to the Y, I (fig. 3.13) and X configurations seen in the light microscope. Some fragments of myofibrils were less damaged (fig. 3.16) and showed the characteristic I bands with their Z lines and A bands but the H lines were not visible. The Z lines, where they were present, were associated with tubular remnants of sarcoplasmic reticulum (fig. 3.16).

The inter-myofibril spaces, which in some regions were enlarged (fig. 3.16) contained injured mitochondria, vacuoles, myelin-like figures and other sarcoplasmic debris (fig. 3.12, 3.14, 3.15). The myelin figures were irregular structures of concentrically arranged, paired membranes (fig. 3.13, 3.14). The electron dense, pairs of membranes were separated from each other by an electron translucent zone 3·5 nm wide (fig. 3.14). The distance between the two membranes of one pair was 2·0 nm wide (fig. 3.14). The membranes of the myelin figures were continuous with the tubular sarcoplasmic reticulum (fig. 3.17). The membranes which constitute the myelin figures were thicker than those of the sarcoplasmic reticulum (fig. 3.17).

The mitochondria showed various responses to the

treatment (fig. 3.13, 3.18, 3.19). Some mitochondria showed loss of cristae (fig. 3.18), in some the outer membrane had ruptured (fig. 3.13) whilst others contained electron dense, needle-like particles (fig. 3.13, 3.19). The needle-like particles were surrounded by granular material (fig. 3.19) which occasionally contained circular electron dense bodies. Undamaged cristae were also present in the mitochondria which contained the needle-like particles.

The sarcoplasm in some regions was very sparse, containing only a few remnants of sarcoplasmic reticulum and scattered ribosomes (fig. 3.16, 3.17). In other regions the sarcoplasmic ground substance was condensed (fig. 3.19, 3.20), glycogen particles were still present in the sarcoplasm in some regions (fig. 3.20). The sarcoplasmic nuclei were frequently compressed and elongated (fig. 3.20).

The sarcolemma membrane was ruptured and often separated from the basement membrane complex (fig. 3.13, 3.19). The basement membrane complex was disrupted and occasionally it had ruptured (fig. 3.13, 3.19). The basement membrane complex had ballooned away from the myofibrils in several places (fig. 3.13, 3.19). The basement membrane complex had sustained less injury than the sarcolemma membrane during the treatment.

The inter muscle fibre spaces were enlarged (fig. 3.19, 3.21) and contained cell debris, injured blood capillaries, membrane bound vesicles of various sizes and densities

degenerate nuclei and condensed cytoplasmic ground substance. The collagen fibres of the endomysium showed no distortion and no loss of the characteristic banding (fig. 3.22).

ii) Transitional zone

One of the most conspicuous features of the transitional zone 20 minutes after treatment was the oedema in the wide inter-muscle fibre spaces (fig. 3.23). The oedema contained degenerate cell organelles, injured blood capillaries and vesicles.

The muscle fibres showed signs of severe contraction, rupture and loss of normal transverse banding but was not as extensive as in the central zone (fig. 3.23). Some of the muscle cells were less injured and showed normal banding (fig. 3.24).

The mitochondria were swollen and degenerate throughout the transitional zone (fig. 3.23) and few scattered myelin-like figures were seen. Fewer sarcolemma nuclei in the transitional zone showed extreme degenerative changes (fig. 3.23).

The sarcolemma membrane also demonstrated various degrees of response to the treatment. In some regions of the cell the sarcolemma membrane was intact, in others it had ruptured (fig. 3.24). The basement membrane complex of the endomysial tube was largely intact (fig. 3.23, 3.24).

The satellite cells were seen in the normal position (fig. 3.23) juxtaposed to the muscle fibre. The mitochondria however, were affected (fig. 3.23) and had lost

their internal structure.

iii) Peripheral zone

The general impression of the peripheral zone 20 minutes after treatment was of normal tissue, although some of the mitochondria were swollen with a loss of cristae (fig. 3.25).

C Two hours after treatment

i) Central zone

Two hours after treatment the muscle cells, in the central zone, showed further degeneration. The degeneration had progressed from severe contraction at 20 minutes post treatment to a picture of destruction and fragmentation of the myofilaments (fig. 3.26, 3.27). The myofibril structure had broken down (fig. 2.26, 2.27, 2.28) leaving clumps of myofilament fragments interspersed with myelin figures, remnants of sarcoplasmic reticulum, degenerate mitochondria and floccular material. Where the myofibril injury was not so extreme the myofilaments disintegration occurred at the ruptured ends of the myofibrils (fig. 3.29).

The numerous myelin figures mainly occurred in groups and showed close associations with the remnants of sarcoplasmic reticulum (fig. 3.30). The rows of small circular structures (each with a diameter of 20 nm) (fig. 3.28) and stacks of electron dense membranes (fig. 3.28) indicated that the myelin figures were cylindrical.

The sarcolemma nuclei were highly degenerate (fig. 3.31) and irregular. The nuclei were scattered amongst the muscle debris and were not in the normal peripheral position

(fig. 3.26). The chromatin was condensed and very electron dense.

The sarcolemma membrane was disrupted and only small remnants were found juxtaposed to the basement membrane complex of the endomysial tube (fig. 3.28, 3.32). The basement membrane complex was very contorted (fig. 3.26). The basal lamina and its associated collagen showed a resistance to the treatment (fig. 3.32) frequently being the only recognisable portion of what was once a muscle cell. Large portions of endomysial tubes were devoid of muscle cell structures (fig. 3.32). The contorted "empty" endomysial tubes contained only floccular material and scattered vesicles (fig. 3.32).

The muscle cells and "empty" endomysial tubes were surrounded by oedematous fluid, which contained a few erythrocytes, cell debris, polymorphs (fig. 3.33) and disposed floccular material. Both the inter and intracellular floccular material had the same appearance (fig. 3.33, 3.32, 3.28).

The satellite cells, which could only be recognised in the periphery of this zone, were also severely affected (fig. 3.34). The satellite cells showed a degenerate nucleus with an almost total loss of nuclear membrane and rupture of the cytoplasmic membrane (fig. 3.34).

ii) Transitional zone

The pathological changes in the muscle cells in the transitional zone were varied but in general the muscle cells nearest the central zone showed the most severe changes.

The intercellular spaces were wide and were filled with oedematous fluid (fig. 3.35, 3.36, 3.37). The myofibrils showed different degrees of injury but none as severe as in the central zone (fig. 3.35, 3.36, 3.37). In the same muscle cell the myofibrils in some regions showed a complete loss of transverse banding and in other regions the myofibrils showed a series of similar electron dense bands (fig. 3.35). In other muscle cells the myofibrils demonstrated normal transverse banding patterns but the myofibrils themselves were disorganised to different degrees (fig. 3.36, 3.37). In the less injured muscle cells in this zone the most conspicuous feature was the large numbers of glycogen particles (fig. 3.37) interspersed between the myofibrils. Sarcolemma nuclei showed different reactions and some had migrated from their normal peripheral positions towards the central regions of the cell. These nuclei were surrounded by glycogen particles.

iii) Peripheral zone

The muscle cells in this zone were swollen but did not show conspicuous changes from 20 minutes to two hours post treatment, apart from a slight increase in the numbers of glycogen particles.

D Twenty four hours after cryosurgery

i) Central zone

By 24 hours after treatment the normal structure of the muscle cells in the central zone had been completely lost (fig. 3.38, 3.39). The myofibrils had disintegrated

into irregular masses in which small myofilament fragments could be detected (fig. 3.38, 3.39). These were extensive maize-like accumulations of sarcoplasmic reticulum interspersed between areas of irregular degenerate myofibrils (fig. 3.40). Myelin figures were found amongst the sarcoplasmic reticulum but in general there were fewer myelin figures in the lesion than at 20 minutes and two hours post treatment. The mitochondria were easily discernable (fig. 3.38, 3.39) as they contained numerous swollen cristae. The mitochondria also contained circular, electron-dense bodies approximately 0·13 nm in diameter (fig. 3.38).

Despite the excessive destruction within the myotube the basal lamina of the basement membrane complex remained relatively intact (fig. 3.38, 3.40). However, only small fragments of the sarcoplasmic membrane remained (fig. 3.38).

There were large areas in the myotube containing only floccular matrix and scattered membrane bound vesicles (fig. 3.40, 3.41).

One of the most conspicuous changes in the central zone 24 hours after treatment was the large numbers of leucocytes in the lesion (fig. 3.41). The leucocytes have the appearance of neutrophil polymorphonuclear leucocytes (or polymorphs) with multilobular nuclei and many, small membrane bound cytoplasmic vesicles (fig. 3.39, 3.41). The polymorphs were seen in large numbers outside the endomysial tube (fig. 3.41) and in some places they had penetrated the endomysial tube and were seen juxtaposed

to the sarcoplasmic debris (fig. 3.39). Some polymorphs had extended pseudopedia, which were devoid of organelles. The cytoplasmic vesicles were of various sizes and their contents varied from electron translucent to extremely electron dense (fig. 3.39, 3.41, 3.42). The polymorphs were frequently accompanied by lymphocytes and erythrocytes (fig. 3.41).

The muscle cell nuclei were degenerate, rounded and scattered randomly in the sarcoplasmic debris (fig. 3.43).

The extra cellular spaces were filled with oedematous fluid (fig. 3.43) containing numerous vesicles, degenerate blood capillaries and collagen fibre fragments (fig. 3.40, 3.43). As well as the polymorphs, lymphocytes and erythrocytes, there were degenerate cells in the inter-cellular oedema (fig. 3.44). These degenerate cells contained numerous lysosomal and autophagic vacuoles and their cell membranes were ruptured (fig. 3.44).

ii) Transitional zone

The transitional zone 24 hours after treatment showed a range of pathological changes. Some of the extremely damaged muscle fibres showed the same features as those in the central zone (fig. 3.45). The endomysial tube had been invaded by a polymorph which was seen adjacent to the round mitochondria (fig. 3.45). Interspersed between the mitochondria were small vesicles, coagulated material (fig. 3.45) and remnants of myofilaments. The endomysial tube was intact but the sarcolemma membrane had disappeared.

Other muscle cells contained ruptured myofibrils (fig. 3.46) but the fragments still showed the normal banding. The main feature of these less injured cells was the change in the position of the nuclei (fig. 3.46). The nuclei were no longer seen in the usual peripheral position but were in the centre of the cell (fig. 3.46). The migrated nuclei had a normal appearance although they were less elongated, and the nuclear membrane was intact (fig. 4.36). There were no mitochondria associated with these nuclei, only scattered ribosomes (fig. 3.46).

The satellite cells (fig. 3.47) in the transitional zone were relatively undamaged even though the associated muscle fibre showed considerable changes. There was evidence of separation of the satellite cell from the normally close association with the muscle cell (fig. 3.47, 3.48). The separation was associated with a break in the sarcolemma (fig. 3.47) and also of the endomysial tube (fig. 3.48).

The intercellular spaces were either narrow (fig. 3.47) and contained no oedematous fluid, or wide (fig. 3.45, 3.49) and filled with oedematous fluid, various cells, cell debris and scattered collagen fibres.

iii) Peripheral zone

The peripheral zone showed no further noteworthy changes at 24 hours post treatment.

Summary of Results

1 Twenty minutes after treatment

Three zones could be distinguished in the lesion. The central zone showed the most cellular damage. The myofibrils, sarcoplasmic reticulum and other tubular systems as well as the mitochondria and the sarcolemma nuclei were damaged. The majority of the basal lamina was intact and showed a good deal of resistance to the freezing episode. Towards the periphery the cellular damage was less, although some of the muscle cells showed evidence of contraction, whilst others were partially injured. The endomysial connective tissue contained oedematous fluid and degenerated fibroblasts. The blood vessels contained degenerate endothelial cells. The damage to the endomysium decreased towards the peripheral area.

2 Two hours after treatment

The cells of the central zone showed increased disruption and disorganisation of their intracellular components. The satellite cells could be recognised at the periphery of the central zone. Towards the transitional zone, the cellular damage and intensity was varied. The oedematous fluid had increased and contained many polymorphs.

3 Twenty four hours after treatment

The cells of the central zone were in an extreme state of degeneration.

The basal lamina was still intact and there was a large infiltration of the polymorphs, most of which surrounded the degenerate muscle cells. Towards the transitional zone, the muscle cells showed a wide range of degenerative changes in which some of the cells were fatally injured whilst others were partially damaged. The satellite cells associated with the muscle cells of the transitional zone were only slightly affected. Many satellite cells showed evidence of active separation from the muscle cells.

Discussion

a) Central zone

The necrosis of the muscle fibres after cryosurgery was observed in the light microscope. The electron microscope enabled the progressive destruction of the muscle fibres to be observed in greater detail.

Possible causes of injury to the muscle tissue, as a result of cryosurgery, are the formation of ice crystals, severe contraction of the fibres and ischemia. It was difficult to assess the full extent of any damage due to ice crystal formation as the first biopsy was taken 20 minutes after treatment, by which time any ice crystals would have melted. Small intracellular ice crystals have been reported to cause a complete loss of structural detail in muscle fibres (Whittaker, 1974b). Whereas the large intracellular ice crystals seen by Whittaker (1974b) in muscle cells caused compression of the normal architecture but the myofilaments and mitochondria remained intact. The present observations were more consistent with the formation of large intracellular ice crystals which would have formed on slow thawing after fast freezing (chapter 1). However, Menz (1971) observed that the growth of large ice crystals caused the greatest amount of disruption of sarcoplasmic reticulum and mitochondria. It is difficult to imaging how the lace-like appearance of cell contents interspersed between extensive ice crystals in Whittaker's (1974b) photographs could reorganise themselves on thawing to give the reasonably regular appearance of the muscle cells

in the present study 20 minutes after treatment. The whole scale destruction in the tissue by ice crystals was not evident in the present study. One of the possible explanations for the difference in the two studies is that Whittaker (1974b) applied the cryoprobe to the overlying epithelium, whereas in this study the cryoprobe was applied directly to the muscle. Low temperature may have been achieved more rapidly when the probe was applied directly to the muscle. Epithelium is a protective tissue and in fulfilling this role may have reduced the cooling rate in the underlying muscle. The techniques of freeze substitution may also have lead to an exaggerated appearance of damage. Evidence of ice crystal damage to muscle one minute after thawing has been seen in the sarcolemma nuclei (Whittaker, 1975).

It is thought that the major cell damage, in the present study, was due to severe contraction. The violent contraction could have occurred at the moment of cold application or during thaw. Twenty minutes after treatment the majority of the Z-lines in the muscle fibres were destroyed or severely distorted. Normal contraction involves less shortening of the myofilaments and a reversible change not seen in the present study. Muscle fibres could be stretched, after extreme contraction caused by Faradization and gradually brought back to their original length (Nageotte, 1937). The muscle fibre could be contracted a number of times before there was complete failure of the contractile mechanism. Faradization did

not cause obliteration of the Z-lines (Nageotte, 1937), merely brought them closer together. Nageotte (1937) also showed that frozen, isolated muscle underwent sudden extreme contraction on thawing, which was irreversible. The observations, 20 minutes after freezing indicated that damage to the Z-lines was a result of irreversible contraction. Huxley and Taylor (1955) stated that the Z-lines were the bases against which contraction was effected and so it would seem logical to assume that violent contraction was responsible for Z-line injury.

Associated with the severe contraction in the study were the myelin figures. The myelin figures are thought to be damaged sarcoplasmic reticulum and not fixation artefacts (Rash, Gey and Gey, 1970). Garry and Henry (1971) concluded that myelin figures were normal cell components as they were found in fixed and unfixed fungal material. There were no myelin figures in the normal muscle observed in this study, only in the injured. The numbers of myelin figures was directly proportional to the degree of muscle cell injury. Twenty four hours after denervation, myelin figures were seen in rat muscle (Manolov and Ovatscharoff,1974). Price, Howes and Blumberg (1964a) concluded that myelin figures were a direct result of damage to the membranous systems of the muscle cells and also of salt accumulation within damaged cells leading to hydration of some of the lipid components. The results showed a direct association of myelin figures with the sarcoplasmic reticulum and not with the mitochondria as

seen by Whittaker (1975). The sarcoplasmic reticulum is intimately related to the Z-lines (Adams, Brown and Pearson, 1962). It is postulated that when the Z-lines were destroyed by the extreme contraction, the sarcoplasmic reticulum membranes normally under tension, would suffer a recoil-like reaction. This would produce the spirals of thickened membranes. Where the Z-lines were still recognisable remnants of sarcoplasmic reticulum were attached. It is difficult to know which came first, the damage to the sarcoplasmic reticulum or the myofibrils. Menz (1970) speculated that the release of calcium from injured sarcoplasmic reticulum would trigger the contraction. The injured muscle cells would soon have been unable to metabolise excess calcium released by the S R and this would have led to an increase in cell injury. The sarcoplasmic reticulum is also thought to be involved with transmitting nerve impulses and would have been involved with any nerve impulses concerned with the fatal contraction.

The changes in the muscle fibres caused by experimental ischemia were very similar to those produced by extreme cold (Adams, 1975). In the present study ischemia is not thought to have caused the fatal injury to the muscle cells. The fatal changes in the muscle cells were seen before the full effects of ischemia could have been manifested (Adams, 1975; Whittaker, 1975). Degeneration of two lines is not usually apparent until 24 hours after torniquet removal (Stenger, 1962).

Differences in the microcirculation surrounding muscle fibres has been thought (Whittaker, 1974b) to be responsible for the variation in ice crystal damage to muscle cells. This idea is based on evidence that excised muscle fibres, with no blood supply, contain smaller and more regular ice crystals than the *in vivo* muscle. It is thought that this is not the complete explanation as other factors such as glycogen status, cell pH, nerve supply and muscle cell type may all contribute to variation in degree of injury. Excised muscle will be frozen from all surfaces of the block, whereas *in vivo* the freezing occurred from one direction only.

The mitochondria displayed various responses to the freezing episode 20 minutes after treatment. Some of the mitochondria contained fine needle-like structures which were not present at two hours post treatment. Para-crystalline particles have been reported by Hanzlikova and Schiaffino (1977) after ischemia in rat skeletal muscle. The crystalline-like inclusions have been shown to be proteinaceous in nature (Hanzlikova *et al*, 1977) and it was suggested that they formed from the polymerization of different enzymes in the intermembranous spaces of the mitochondria.

The appearance of the mitochondria two hours post treatment was the same as those observed by Whittaker (1975). The mitochondria shows three types of appearance and were either grossly swollen and represented only by a double limited membrane; were slightly swollen with relatively

intact cristae or were small and dense with a condensed matrix. By 24 hours the first type, which were situated between the myofibrils at two hours post operatively, had disappeared.

The freezing episode was fatal to some of the mitochondria and this would mean a failure of muscle cell function. Other mitochondria showed more restriction to the treatment or were able, to some extent, to restore their membranes and also, possibly, some mitochondrial function. Twenty-four hours post treatment the mitochondria, in otherwise severely disrupted muscle cells, had complete outer membranes and contained numerous cristae. These mitochondria did not have a normal appearance and their functional status was unknown. The mitochondria of mouse liver cells distorted by riboflavin deficiency were shown to return to normal size and function within several hours (Bernard, Robert and Wynder, 1968). The potential of mitochondria to regenerate in muscle cells after low temperature treatment requires much further study.

The freezing and subsequent thawing induced typical pathological changes in the sarcolemma nuclei, such as chromatin condensation and disruption of the nuclear membranes with formation of perinuclear spaces. These changes were similar to those reported by Gill and Fraser (1968) after freezing of tissue. In many cells the sarcolemma nuclei were ruptured or severely compressed. The abnormally elongated nuclei were located between bands of myofibrils and the basement membrane complex. The

compression may have been due to the force of the occulent muscle fibre contraction or to displacement by the formation of large ice crystals (Whittaker, 1974b). Ice crystal spaces were reported in the sarcolemma nuclei one minute after thawing by Whittaker (1975). By two hours post treatment highly degenerate nuclei were found scattered throughout the cells, the movement of these nuclei was thought to be passive and a result of loss of cell turgor and integrity. If nuclei remained juxtaposed to remnants of the sarcolemma membrane they are reported to show less degenerative changes and survive the trauma (Adams, 1975). These observations are consistent with those of the present study. The injured sarcolemma nuclei in the severely damaged muscle cells showed progressive disintegration from 20 minutes to 24 hours.

The sarcolemma membrane showed progressive dissolution from 20 minutes to 24 hours after treatment. The portions of sarcolemma membrane that were seen at 20 minutes seemed to disappear completely by 24 hours, leaving only the lamina densa and collagen fibres of the basement membrane complex. The resistance of the lamina densa to injury has been noted following severe cold, trauma or ischaemia (Albrook, 1962; Whittaker, 1975). Collagen fibres were seen to be particularly resistant to damage by extreme cold, as reported by Lincoff and McLean (1965). The association of the collagen fibres with the lamina densa is suggested as a possible explanation for the good preservation of the structure. In other tissues, for example, the oral

epithelium (Tyldesley and Kempson, 1976), the first signs of pathological change are seen as a breakdown in the basement membrane complex. The freezing episode appears initially to cause mechanical injury to which the basal lamina is resistant.

Experimental injury such as ischemia (Stock et al., 1973) nitrite injection (Dynarowigz, Wilczek and Garbulinski, 1975) caused hydrolysis of glycogen several hours after treatment. Glycolysis also increases during changes in cell pH (Bandall, 1961) and the reduction of glycogen during muscle ischemia is thought to be due to the mobilization of glycogen during contraction (Scully, Shannon and Dickresin, 1961). The reduction in muscle cell glycogen due to freezing may have been in response to changes in pH or to the severe contraction. Whittaker (1975) reported a complete loss of glycogen one hour after freezing.

The dissolution of the myofilaments was progressive, the first sign on fatal injury was the rupture of the myofibrils followed by disintegration of the individual myofilaments. The fragmentation of the myofilaments at the broken ends of the myofibrils was seen at two hours post treatment. By 24 hours post treatment the disintegration of the myofilaments was almost complete giving floccular masses within the cell. Ischemia and poisoning have also produced fragmentation and dissolution of the myofilaments (Miledi and Slater, 1969). The onset of these changes is more rapid in the frozen tissue.

The progressive disintegration and lysis of the muscle cell after dystrophic and atrophic changes have been shown to be related to the increase in hydrolytic enzyme activity (Weinstock and Iodice, 1969). The activity of hydrolytic enzymes is directly proportional to the severity of muscle damage (Pearson and Kar, 1971). The increase in myofilament fragmentation and the decrease in numbers of myelin figures by 24 hours is attributed to the general action of hydrolytic enzymes.

By 24 hours after treatment the central zone contained large numbers of polymorphonuclear leucocytes and lymphocytes. Some of these phagocytic cells had penetrated the endomysial tubes of the damaged muscle cells. Yanko, Behar and Yarom (1974) also observed the rapid infiltration of large numbers of polymorphs after thawing and freezing rabbit extraoccular muscle. The appearance of phagocytes so quickly after injury was thought to have some influence on the speed of regeneration (Yanko et al., 1974). The polymorphs contribute to cell debris removal as their polysomes contain hydrolytic enzymes (Cheville, 1976).

The oedematous fluid increased rapidly in the first two hours after treatment but by 24 hours there was a decrease. The faster appearance of oedematous fluid after cryosurgery than after other types of tissue injury was also noted by Poswillow (1971b). The decrease in oedematous fluid by 24 hours was thought to be associated with the disorganisation of the connective tissue.

The satellite cells also showed progressive,

degenerative changes. Even though the muscle cells were severely injured the satellite cells were still bound by the intact basement membrane complex, at two hours post operatively. The progressive pathological changes have been thought to be related to the injury to the satellite cell membrane (Litvan, 1972; Whittaker, 1975). By 24 hours it was not possible to identify satellite cells in the central zone.

b) Transitional and Peripheral zones

The general impression of the effects of the cryoprobe on the transitional zone was of less overall injury to the tissue but that there was a greater variation in the extent of the injury to individual cells. There are several possible explanations for this appearance. If a cell is not in direct contact with the cryoprobe its chances of survival are better, this is to be expected and has been demonstrated by Mazur (1968). Whittaker (1974b) showed that ice crystals formed mainly in the extracellular spaces in the peripheral areas. Extracellular ice crystals were not so damaging to the tissue (Whittaker, 1974b). The effect of intra cellular ice crystals in some of the cells in this region was difficult to assess. The distribution of the fibrous endomysium and paramysium may have caused a differential cooling rate, away from the ice ball. The effect of the distribution of the blood supply may also have had a more significant bearing on the cooling rates in the area peripheral to the ice ball.

The extent of the oedema was greater 20 minutes after treatment, in the transitional zone than in the central zone. Oedema is related to the degree of injury to the small blood vessels. Freezing treatment affects the fine physiological balance between the blood capillaries and tissue fluids (Meyer, Hammond and Ketham, 1970; Whittaker, 1973). The endothelial cell injury was greater in venules than the arterioles (Poswillow, 1971). The central zone blood capillaries were so severely injured that little or no oedema resulted. Indeed it is thought likely that the oedema seen in the central zone was derived in the transitional zone, where the blood capillaries produced copious amounts of oedematous fluid.

One of the most significant observations in the transitional zone was the migration of apparently normal sarcolemma nuclei to the central regions of the muscle cells. This migration was most conspicuous 24 hours after treatment. Nuclear migration in muscle cells has also been reported after denervation (Lubelius, Soanesson and Stamenovik, 1970), degenerative changes (Adams, et al., 1962), and administration of local anaesthetics (Beniot, et al., 1970). The nuclei are thought to have been active and were associated with accumulation of ribosomes in the areas between the ruptured myofibrils. This was most probably an indication that protein synthesis and tissue repair processes were taking place.

By two hours after treatment the glycogen particles had increased in the transitional zone. The effect of

cold on the metabolic activity of the enzymes associated with glycogen is unknown. Fast freezing and slow thawing of bone marrow block in vitro resulted in deminished enzyme activity (Melnick, 1968). The increase in glycogen particles indicated that the cells had not suffered ischemia as glycogen was shown to decrease after acute arterial occlusion. Adolfson (1973) showed an initial reduction in muscle cell glycogen 15 minutes after electrical stimulation, followed by an increase in glycogen synthesis. Hypermobility of muscle cells achieved by exercise also increases glycogen synthesis (Canal and Frattola, 1970). In the transitional zone the muscle cells may have responded to the activity caused by the freeze-thaw cycle by glycogen production in some of the cells. This response indicates that some of the normal metabolic mechanisms were still intact.

Adrenalin has been shown to effect glycogen synthesis in rat skeletal muscle (Popova, Orlova and Rosenffed, 1969). It is possible that the stress experienced during the inter-operative period would have stimulated adrenalin production and subsequent glycogen synthesis.

By 24 hours the satellite cells in the transitional zone had increased in size and had started to separate from the parent cell. These changes in the satellite cells were reported in muscle tissue after denervation but only after 1 week post treatment (Hess and Rosner, 1970). The factors influencing the separation of the satellite cells was unknown.

The observations on the degenerative changes in the muscle cells have raised many questions and further studies are required to elucidate these problems.

CHAPTER 4

ULTRASTRUCTURAL OBSERVATIONS OF THE REPARATIVE CHANGES IN MUSCLE TISSUE AFTER CRYOSURGERY

Introduction

The previous chapter described ultrastructural observations of the degeneration of muscle after cryosurgery. A study of the course of the degeneration of muscle after injury is vital to the understanding of the subsequent regeneration. The clinician is primarily concerned with the speed and extent of regeneration after injury, particularly the recovery after surgically produced lesions. The regeneration of muscle observed in the electron microscope is described in this chapter.

Materials and Methods

i) Surgical procedures

As in chapter 2 materials and methods, section (i).

ii) Electron microscopy

As in chapter 3 materials and methods, section (ii).

Observations

The regeneration of muscle cells seen in the light microscope was also observed in the electron microscope. The regeneration of the muscle cells was continuous and discontinuous (Discussion, chapter 2). The continuous regeneration was seen in the partially damaged muscle fibres of the transitional zone. The regeneration occurred

as myofilament growth in the centrally directed end of the injured muscle cells. The discontinuous regeneration was seen in the peripheral half of the central zone and had two different forms. Discontinuous regeneration type 1 consisted of the formation of new myoblasts within old endomysial tubes. The myoblasts fused to form myotubes. Discontinuous regeneration type 2 consisted of newly formed myoblasts which had no association with an old endomysial tube.

A One week after cryosurgery

The lesion one week after cryosurgery showed much evidence of reparative changes. The first signs of muscle cell regeneration were seen at this time. The biopsy could still be divided into three zones for descriptive purposes, the central, transitional and peripheral zones. As the peripheral zone did not show significant differences from the 24 hour biopsy, only the central and transitional zones will be reported.

(i) Central zone

The centre of the lesion consisted of a core of necrotic, high degenerate cells (fig. 4.1). The outline of degenerate cells could be distinguished but the exact identification of these cells was not possible (fig. 4.1). Surrounding the zone of necrotic debris was a region of tightly packed, phagocytic cells (fig. 4.2). The phagocytic cells, the majority of which were polymorphs, contained numerous heterophagic vacuoles in various stages of digestion

(fig. 4.2). One polymorph showed a membrane bound vesicle containing a whole nucleus (fig. 4.2). Some of the polymorphs contained primary lysosomes (fig. 4.2) whilst in others the cytoplasm was packed with secondary lysosomes. Interspersed between the polymorphs were degenerate cells, cytoplasmic debris, numerous varied vesicles, nuclei at different stages of disintegration and moribund muscle cells. Degenerate muscle cells were recognisable.

The totally necrotic muscle cells in the centre of the lesion were no longer recognisable but the less injured cells could be identified (fig. 4.3). These muscle cells contained distinct myofilaments (fig. 4.3) but there was no transverse banding or normal myofibril formation. There was no evidence of either the sarcolemma membrane or the basement membrane complex (fig. 4.3, 4.4). The damaged muscle cells were surrounded by densely packed polymorphs, other phagocytes and cell debris (fig. 4.3, 4.4). Remnants of sarcoplasmic reticulum and sarcolemma nuclei were in direct contact with the sparse oedematous fluid and polymorphs. Bacteria had invaded the lesion (fig. 4.5) and were found in the oedematous fluid.

Towards the periphery of the central zone the first signs of muscle cell regeneration were observed (fig. 4.6). Within the irregular, undulating remains of the endomysial tube were newly formed cells (fig. 4.6). The micrograph fig. 4.6 illustrates the discontinuous regeneration type 1. The old endomysial tube, which consisted of the lamina densa and connective tissue components of the

basement membrane complex, was continuous (fig. 4.6). The myotube was only in contact with small portions of the new cells or myoblasts (fig. 4.6). At the points of contact the basal lamina was 0·025 μm away from the myoblast membrane (fig. 4.7), which is the normal width of the lamina lucida. The lamina lucida zone contained floccular material (fig. 4.7) which was also seen associated with the basal lamina not in contact with the cell membrane. Within the endomysial tube were two cells (fig. 4.6) and their adjacent cell membranes were disintegrating showing the process of fusion of myoblasts. The myoblasts contained fine filaments, numerous ribosomes and vesicles of varied appearance (fig. 4.7). The cell membrane of the myoblast was very electron dense for most of its length, apart from small discontinuities (fig. 4.6, 4.7). The myotube was surrounded by randomly orientated collagen fibres, connective tissue matrix and fibroblasts (fig. 4.6).

The early stage of myofibril regeneration was represented by the randomly orientated fine filaments (fig. 4.7). These fine filaments showed a close association with the ribosomes and were 8·0 nm in width (fig. 4.7). Small aggregations of filaments orientated parallel to each other and the long axis of the myotube were scattered amongst the mitochondria, ribosomes and randomly orientated filaments (fig. 4.8). At this stage in the regeneration of the muscle cell, sarcoplasmic reticulum was formed from invaginations from the cell membrane (fig. 4.8). Other aggregations of sarcoplasmic reticulum were also seen

close to the cell membrane (fig. 4.8). The parallel bundles of filaments contained either thick and thin filaments together or just thin filaments (fig. 4.8). Ribosomes clustered around the free ends of filament bundles (fig. 4.9). At a later stage of development there were more bundles of parallel thick and thin filaments (fig. 4.10). Some of the bundles (fig. 4.10) showed dark bands with the same appearance as Z-lines (fig. 4.10). In between the bundles of filaments were many mitochondria, ribosomes, sarcoplasmic reticulum elements and random filaments (fig. 4.10). The nuclei in the regenerating cells were prominent, oval, and situated in the middle of the cell (fig. 4.11). Juxtaposed to the nuclei of some of the myotubes were Golgi bodies surrounded by small vesicles (fig. 4.11).

Another indication of healing processes in the central zone were the presence of numerous active fibroblasts (fig. 4.12). The fibroblasts were surrounded by newly formed collagen fibres (fig. 4.12).

The blood vessels in the regenerating region of the central zone contained large numbers of neutrophils, other polymorphs and a few erythrocytes (fig. 4.13). The endothelial cells were numerous and cuboidal in outline (fig. 4.13). The nuclei of the endothelial cells were large, rounded and surrounded by cytoplasm (fig. 4.13) which contained a few organelles.

(ii) Transitional zone

Reparative processes and new myofilament formation were seen in the less injured cells of the transitional zone (fig. 4.14). Fine filaments were situated between the ends of the myofibrils (fig. 4.14). The areas containing the fine filaments were rich in mitochondria and ribosomes (fig. 4.14). The nucleus was found in the middle of the injured myofibrils (fig. 4.14). The newly formed, fine filaments were frequently closely associated with the sarcoplasmic reticulum (fig. 4.15). One of the sarcoplasmic reticulum cisternae contained fine filamentous structures, with a similar appearance to the newly formed filaments (fig. 4.15). In some cases, the fine, newly formed filaments appeared to have grown from the ruptured edges of the myofibrils (fig. 4.16). The orientation of the fine filaments which emanated from the ruptured myofibrils was parallel to the long axis of the myofibrils (fig. 4.16). These micrographs represented continuous regeneration (fig. 4.14, 4.15, 4.16).

The extent of muscle cell injury was very variable in the transitional zone. One week after treatment there were cells in different stages of regeneration (fig. 4.17). One of the conspicuous features of the transitional zone was the large numbers of collagen fibres. The cells that had sustained greatest injury had large numbers of pinocytic-like vesicles along the sarcolemma membrane (fig. 4.17). These invaginations of the sarcolemma membrane were thought to be early stages of sarcoplasmic

reticulum formation (fig. 4.18).

Golgi bodies, surrounded by numerous vesicles were also present in the perinuclear sarcoplasm of the cells of the transitional zone (fig. 4.19).

B Two weeks after cryosurgery

(i) Central zone

The most central part of the lesion two weeks after cryosurgery was packed with collagen fibres (fig. 4.20); a few macrophages were scattered amongst the fibroblasts.

Peripheral to the area packed with collagen there was a myotube with no obvious association with a remnant endomysial tube containing myoblasts (fig. 4.21). The myotube was surrounded by fibroblasts (fig. 4.21) and collagen fibres. There were numerous mitochondria, ribosomes and lipid-like deposits in the centre of the myotube (fig. 4.21). The bundles of aggregated filaments were situated at the periphery of the cell (fig. 4.21). In some of the regenerating myotubes there was no evidence of sarcoplasmic reticulum only small fragments of rough endoplasmic reticulum (fig. 4.21). This micrograph is an example of discontinuous type 2 regeneration.

By two weeks after treatment the cell membrane of another myotube closely followed the course of the basal lamina (fig. 4.22). The basal lamina appeared to have been newly formed as there was no established association with the collagen fibres. In some cells there were invaginations of the cell membrane (fig. 4.22) which had

the appearance of developing sarcoplasmic reticulum. The myotubes contained scattered fragments of myofibrils (fig. 4.22, 4.23) which contained thick and thin filaments and showed transverse banding (fig. 4.23). In some of the myoblasts the myofibril fragments were surrounded by large numbers of fine filaments and some mitochondria (fig. 4.23). One myotube, more advanced in its development (fig. 4.24) was almost completely filled with myofibrils. The portions of myofibrils demonstrated distinct transverse banding (fig. 4.24). The tips of some muscle cells showed a few complete myofibrils (fig. 4.25), although the myofibrils were not thick they had a normal appearance (fig. 4.25). The sarcolemma membrane had a normal structure but an undulating outline (fig. 4.25).

(ii) Transitional zone

The reparative changes seen in the transitional zone one week after cryosurgery were still present two weeks after treatment (fig. 4.26). There were larger numbers of mitochondria and ribosomes present at the fractured edges of the myofibrils (fig. 4.26). Some of the cells in this region appeared completely normal with a peripheral nucleus and normal myofibrils. The basement membrane complexes of neighbouring cells were frequently only separated by a few collagen fibres and fibroblast cell processes (fig. 4.27).

C Three weeks after cryosurgery

(i) Central zone

The centre of the lesion consisted only of densely packed bundles of collagen fibres (fig. 4.28) and scattered fibroblasts. The collagen fibre bundles were randomly orientated (fig. 4.28).

At the edge of the dense centre most region, regenerating myotubes penetrated the bundles of collagen fibres (fig. 4.29). Some of the myotubes were packed with myofilaments (fig. 4.29), consisting of both thick and thin elements. However, there was no organisation of the myofilaments into myofibrils in some cells, just randomly orientated bundles of thick and thin myofilaments (fig. 4.29). In occasional myotubes there was some evidence of myofibril formation and the presence of cross banding (fig. 4.30). The basal lamina and its accompanying collagen fibres was normal (fig. 4.29, 4.30) and closely followed the cellular outline. Other myotubes showed distinct degenerative changes (fig. 4.31) which included disintegration of the mitochondria, fragmentation of myofilaments and distortion of the nucleus (fig. 4.31). Some myotubes consisted of only floccular material, scattered vesicles and degenerate mitochondria, contained within a normal lamina (fig. 4.32). There were no signs of active myofilament production, just evidence of disruption and disintegration of the myofilaments (fig. 4.33) of the type 2 discontinuous regenerated myotubes.

The most conspicuous feature of the myoblasts in the

central zone was the absence of sarcoplasmic reticulum (fig. 4.29, 4.30, 4.32, 4.33).

Polymorphs were found juxtaposed to degenerate myoblasts towards the periphery of the central zone.

(ii) Transitional zone

In the transitional zone three weeks after treatment the regenerating portions of the partially injured muscle cells had penetrated the bundles of collagen at the edge of the central zone (fig. 4.34). Myofilaments were organised into myofibrils and showed normal transverse banding (fig. 4.34). The free ends of the myofibrils were surrounded by numerous mitochondria. The collagen fibrils showed a conspicuous attachment to the basement membrane complex of the endomysial tube (fig. 4.34). There were blood capillaries on two sides of the muscle cell in figure 4.34. The collagen fibrils were embedded in a dense matrix (fig. 4.34). In the ends of some regenerated muscle cells the old basal lamina had been partially replaced by new basal lamina-like material (fig. 4.35, 4.36). The basal lamina of the old endomysial tube had ballooned away from the cell (fig. 4.35). In another cell the collagen fibres associated with the new basal lamina had formed in some parts (fig. 4.36).

Where there were newly formed filaments in the muscle cells the sarcoplasmic reticulum was complete (fig. 4.37). Well developed triads and sarcoplasmic tubular structures were situated between the newly formed fine filaments, which were all of the same thickness (11·0nm) and were

closely associated with the sarcoplasmic reticulum and triads (fig. 4.38). The new filaments did not show the differentiation into thick and thin filaments. The filaments, formed at the ruptured edges of the myofilaments, were all of the fine type despite the presence of thick myofilaments (fig. 4.39) in the myofibril.

D Four weeks after cryosurgery

The cryolesion four weeks after cryosurgery consisted of a central zone packed with collagen fibre bundles, fibroblasts and a few regenerative muscle fibres. Peripheral to the central zone was a region of completely regenerated muscle fibres.

In the central zone by four weeks after cryosurgery there were large lipid-like deposits scattered amongst the connective tissue elements.

The central zone also contained small blood capillaries.

The muscle cells found at the periphery of the central region were irregular in outline (fig. 4.41). These muscle cells contained easily distinguishable myofibrils with clear transverse banding (fig. 4.41) but the myofibrils were not orientated in the normal regular pattern. The basal lamina and sarcolemma membrane were normal (fig. 4.40).

Further away from the central region the regenerated muscle fibres were thicker and showed different degrees of recovery (fig. 4.42, 4.43).

Some cells showed a normal myofibril structure and arrangement (fig. 4.43) whilst in other cells the myofibrils

were much more irregular (fig. 4.42). There was a zone of sarcoplasm 0·43 µm thick between the myofibrils and sarcolemma membrane in some cells (fig. 4.42) which contained numerous vesicles, autophagic vacuoles and degenerate mitochondria. There were some normal mitochondria interspersed between the myofibrils (fig. 4.42) along with some membrane-bound electron-luscent vesicles. The electron-luscent vesicles were 24·8 nm to 200 nm in diameter. The sarcolemma nucleus (fig. 4.42) was normal. At the periphery of the lesion the muscle cells had fully recovered from the treatment.

Discussion

By one week after treatment active repair of the cryolesion had commenced. In the centre most areas the necrotic tissue was still being removed but by two weeks post operatively scar tissue was being formed. The muscle fibres were regenerating with various degrees of success. Repair of the partially injured muscle fibres was relatively straight forward and was completed by a few weeks post-operatively. The muscle cells which regenerated at the periphery of the scar tissue presented a more complex picture.

The most popular theory of muscle cell regeneration after injury stated two types of cell reaction, the first is continuous regeneration and the second is discontinuous regeneration of muscle cells. Continuous regeneration occurs in partially injured muscle cells where there is an

outgrowth from the intact region of the muscle cell (Goodman, 1958). The new growth of myofilaments and myofibrils fills the empty region of the endomysial tube, after the necrotic debris has been removed by phagocytes. The basal lamina forms an essential template for the regenerating end (Field, 1960; Adams et al., 1962).

Muscle cells which originated from the mono nuclear cells called myoblasts are said to be formed by discontinuous regeneration (Speidel, 1938; Adams et al., 1962). The myoblasts differentiate to form multinuclear cells called myotubues (Speidel, 1938; Adams et al., 1962). It is the origin of the myogenic cells which has caused the greatest debate and indeed the discussions still continue.

Satellite cells in frog muscle were first described in 1961 by Muro. The satellite cell is a mononuclear cell located between the muscle fibre proper and its basement membrane. They represent a population of undifferentiated cells, which theoretically could serve as a cellular reserve and could be mobilised to repair damage to the muscle tissue. The presence of satellite cells rekindled the controversy of whether an infusion of new cells from a normally dormant population is necessary for post-traumatic repair or whether the cells of highly differentiated structures can act on their own. In the latter case a nucleus surrounded by a thin rim of cytoplasm breaks off from the injured muscle cell and serves as the cellular source for the regenerating muscle.

Reznik (1969, 1970) studied muscle regeneration in

the mouse and rabbit after cold injury and he claimed that myogenic cells arise by the breaking away of myonuclei from the damaged muscle fibre. There is considerable doubt over the interpretation of Reznik's observations. Reznik (1969) called the "broken off" mononuclear cells presumptive myoblasts or satellite cells. These cells appeared only after injury and not in the normal muscle (Reznik, 1969, 1970). The presumptive myoblasts described by Reznik (1969) have the same appearance as the satellite cells of normal muscle cells. The investigators who have recognised well defined populations of satellite cells in normal undamaged muscle have almost uniformly considered presumptive myoblasts to represent satellite cells which have become activated by injury to the muscle cell (Carlson, 1973). After muscle denervation, satellite cells were shown to separate from the dystrophic muscle and lie free in the interstitial tissue (Hess and Rosner, 1970; Ontell, 1972; Schultz, 1978). The differentiation of satellite cells into myotubes has been observed by several workers (Church et al., 1966; Teravainen, 1970; Moss and Lebland, 1971; Snow, 1978, and Wakayma, 1979). These observations were supported by studied in vitro by Bischoff (1975). It is interesting to note that to date in vitro studies have failed to observe the production of muscle cells from myonuclei (Pullman and Yeoh, 1978).

The satellite cell population responds to trauma by an apparent increase in numbers and also by structural changes (Teravainen, 1970). The amount of cytoplasm and

numbers of ribosomes, R.E.R. and mitochondria increase (Teravainen, 1970). A further indication of activity of these cells was the appearance of prominent nuclei (Teravainen, 1970). In the present study the discontinuous regeneration of the muscle tissue nearest the centre of the lesion had two forms. The first, Type I was seen one week after injury and consisted of fusion of myoblasts within old endomysial tubes. It is thought that the myoblasts within the old endomysial tubes originated from satellite cells that had survived the injury. There is evidence to suggest that satellite cells are more resistant to injury than the muscle cells (Schmalbruch, 1976). This is consistent with the observations in Chapter 3 where it was proposed that severe contraction was responsible for the damage of some of the muscle cells. Satellite cells are undifferentiated and contain no contractile elements so it is possible for them to have been less affected by the treatment.

The myoblasts had fused within the endomysial tube to form myotubes. Active muscle cell regeneration by the fusion of myoblasts to give myotubes has been reported by Libton and Koingsberg (1972), Allam (1979) and Kaldern and Gihila (1979). Myofilaments were seen within the myotubes.

The second form of discontinuous regeneration, type II, seen in the central region was not seen until two weeks after treatment. The type II myoblasts were not associated with old endomysial tubes but they were developing free within the connective tissue. It could be argued that the old

endomysial tubes had disappeared from around these cells. This is thought to be most unlikely as the old endomysial tube was still present at two weeks in the type I discontinuously regenerating myotubes. Also at three weeks after treatment the new basal lamina associated with the continuously regenerating muscle cells was in process of formation within the old endomysial tube. By three weeks evidence of early basal lamina formation was seen around the type II myoblasts.

The origin of the type II myoblasts is unknown but it is thought that they may arise from satellite cells released from muscle fibres in the transitional zone. If the type II myoblasts do develop from satellite cells from the transitional zone it is unknown whether their migration to the central zone is active or passive.

Maturation of the type II myoblasts reached a certain point of early myofilament formation before they started to regress. It is possible only to speculate on the reasons why the regenerating myoblasts cease their development. It is interesting to note that there was no evidence of sarcoplasmic reticulum formation within the type II myoblasts although there was rough endoplasmic reticulum present which is the fore runner of sarcoplasmic reticulum during embryonic development. The absence of sarcoplasmic reticulum may well have been associated with the absence of innervation. Reinnervation is a prerequisite for optimal maintenance of the newly formed cells (McComas, 1977). Regenerating axons reach the denervated muscle and seek

out the sites of old end plates in order to establish new neuromuscular junctions (Bennett, McLachlan and Taylor, 1973). The absence of a suitable site for a neuromuscular junction may well be the cause of the regression of the developing myoblasts.

It is possible that the environment produced by large numbers of active fibroblasts was unsuitable for the development of myoblasts unprotected by a basal lamina. Indeed it has been proposed by Vracko and Benditt (1972) that the basal lamina was important in limiting the extent of connective tissue regeneration. The developing scar tissue may also form a physical barrier to the myotube.

It has been shown (Zenela, 1962) that without reinnervation, normal morphogenesis of muscle is halted short of maturation. Myoblasts and myotubes can proliferate without the need of a nerve supply, but not mature.

The work of Vracko and Benditt (1972) showed new basal lamina production one week after cold treatment. This was much earlier than in the present study as it was three weeks before there was evidence of new basal lamina formation. This inconsistency is thought to be due to the much colder temperature used in the present study. The aluminium disc cooled with CO_2 gas (Vracko and Benditt, 1972) did not give rise to such extensive injury. The basal lamina and the endomysial tube were responsible for the orientation of muscle fibres (Vracko and Benditt, 1972).

By the end of the experiment, the centre most region

of the lesion contained only scar tissue. The large increase in fibroblasts occurred by two weeks post operatively by which time the debris had been removed by hydrolysis (Martinez et al., 1976) or phagocytes. The largest numbers of phagocytes were present one week after treatment presumably attracted by the extensive injury as the bacterial infection was only slight.

In the transitional zone, where there was less injury to the muscle fibres, the cells showed continuous regeneration. Continuous regeneration of injured muscle cells has been reported by Godman (1958) and Field (1960). The regeneration of partially injured muscle fibres was first noticed one week after treatment at the same time as the new myoblasts were seen in the empty endomysial tubes.

The myofibril repair was associated with the migration of sarcolemma nuclei to the centre of the muscle fibre. This was accompanied by an increase in numbers of ribosomes. It has been shown that in chick embryo skeletal muscle that the large polyribosomes were responsible for the myosin synthesis (Heywood, Dowben and Rich, 1967). The contractile proteins were formed in the small ribosomes (Heywood et al., 1967). The presence of large numbers of ribosomes clustered around the free ends of the myofibrils were involved with synthesis of new myofibril elements (Fischmann, 1972).

In the areas where myofilament formation was involved in bridging the gap between two ends of a ruptured myofibril, sarcoplasmic reticulum elements were present. These

were immature elements not unlike the rough surfaced tubular system seen in 10 day cultured chick embryo skeletal muscle (Ezerman and Ishikawa, 1967). In the early studies of continuous regeneration the question of whether the new sarcoplasmic reticulum was derived from the existing sarcoplasmic reticulum or de novo within the cell, was debated when Goria (1972) showed sarcoplasmic reticulum and the T system proliferating, de novo, after denervation: the possibility of both origins was accepted.

In the developing muscle cells the appearance of sarcoplasmic reticulum and the T system preceed the formation of the fine actin filaments (Threadgold, 1976). The Z-lines become established first in order to form an anchor for filament sliding (Threadgold, 1976). These stages were seen in the present observations on continuous regeneration.

By the second week after treatment the muscle fibres showing continuous regeneration contained a large increase in the number of mitochondria. The increase is thought to be due to the increased demand for energy by the regenerating muscle. It has been well documented that the mitochondria are involved with oxidative phosphorylation (Walker and Bert, 1969).

The exact mechanism of assembly of the myofilaments is still not fully understood. The studies of Vracko and Benditt have indicated the importance of the basal lamina in the orientation of newly formed myofibrils. Whether the influence comes from the basal lamina alone

or from an association of the cell sarcoplasmic reticulum is still a matter for speculation.

It was interesting to note the accumulation of lysosomal residual bodies in the repaired cells. It is thought that these bodies originated from either non-digested, necrotic particles or as a byproduct of the repair process. The active degradation of unwanted components could be completed within the autophagic vacuoles with no detriment to the cell (De Duve, 1967).

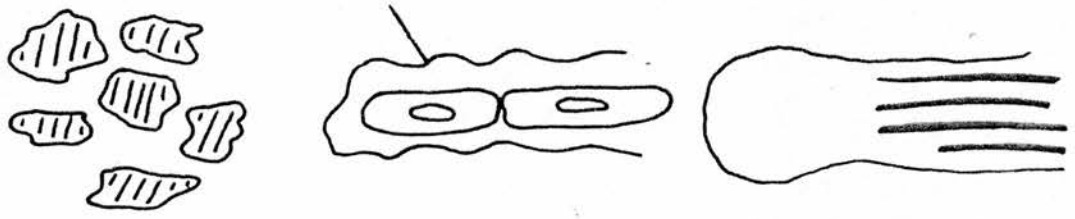
The regeneration of muscle cells after cryosurgery raised many interesting questions and further studies would be rewarding and most beneficial to the understanding of this process.

SUMMARY OF RESULTS - POST TREATMENT

CENTRE

TRANSITIONAL ZONE

1 WEEK

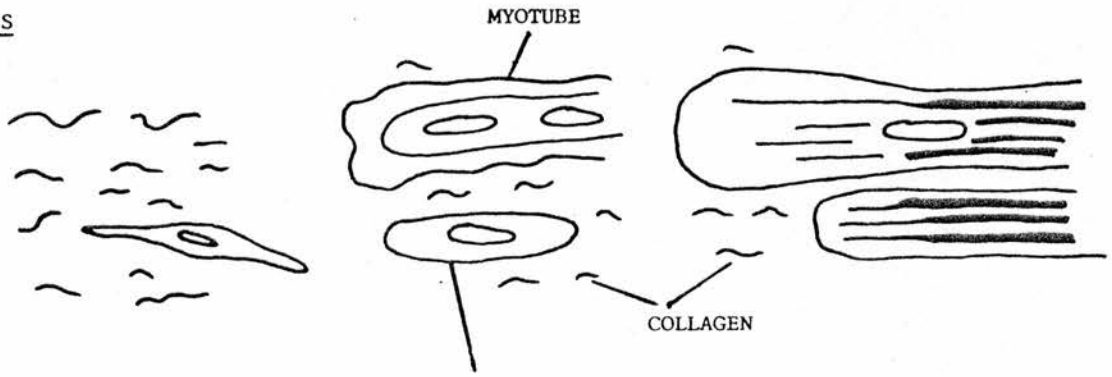


DEBRIS &
PHAGOCYTES

DISCONTINUOUS
REGENERATION
TYPE I

CONTINUOUS
REGENERATION

2 WEEKS

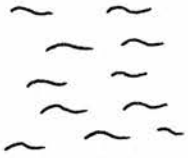


ACTIVE FIBROBLASTS
& COLLAGEN FIBRES

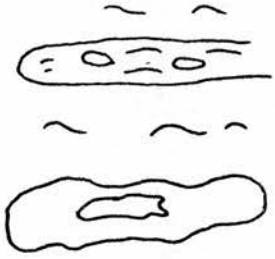
DISCONTINUOUS
REGENERATION
TYPE II

CONTINUOUS
REGENERATION

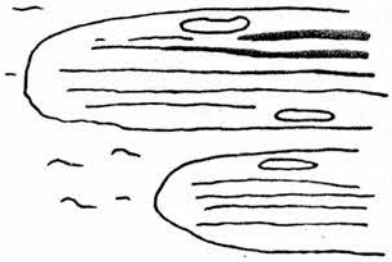
3 WEEKS



COLLAGEN SCAR TISSUE



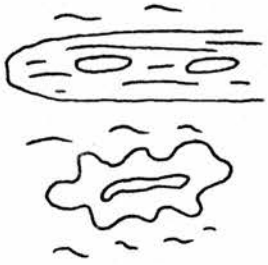
DISINTEGRATION OF REGENERATED MUSCLE CELL



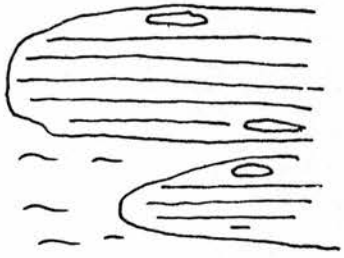
NUCLEI RETURN TO NORMAL POSITION IN REGENERATED MUSCLE CELL

4 WEEKS

SCAR TISSUE



FURTHER DISINTEGRATION OF NEWLY FORMED MYOBLASTS



NORMAL MUSCLE CELLS

CHAPTER 5

LIGHT MICROSCOPIC OBSERVATIONS ON THE EFFECT OF CRYOSURGERY ON THE SCIATIC NERVE OF THE GUINEA PIG

Introduction

Many studies have shown that cold application is a useful technique in the study of neuropathophysiology. Cold application has been an acceptable means of investigating the structure and function of peripheral nerves for many years. As early as 1883 Openchowski showed that local freezing could produce a local discrete lesion in peripheral nerves. Trendelenburg (1917) used ethyl chloride to freeze the phrenic nerve of the dog and found that interruption of nerve conduction by cold would be followed by regeneration and functional recovery.

More recently local freezing of nervous tissue proved to be a useful technique because the cell destruction was localised and produced no haemorrhage (Holden, 1975). There was no neuroma formation after freezing of peripheral nerves (Lloyd, 1977).

The pathological changes associated with nerve freezing were first described by Denny-Brown in 1945. The frozen area degenerates and is followed by regeneration (Denny-Brown et al., 1945; Armstrong, 1959).

Many investigations have shown that one of the benefits of cryosurgery is to produce local nerve blocks (Bernstein, 1963; Feder and Stratigos, 1971) and so consequently it has been used for painless treatment of superficial lesions. Not

all workers agree with the painless aspect of the procedure and state that a local anaesthetic should be used before cryosurgery (Goldstein, 1972; Holden, 1975). The controversy over the degree of pain involved in cryosurgery is still active.

Although the effect of low temperature on peripheral nerves has been studied (Carter et al., 1972; Whittaker, 1974) the knowledge of the degeneration and regeneration of nerves is still incomplete. The present study was designed to observe the pathological changes and regeneration of a peripheral nerve in both the light and electron microscopes. A maximum recovery time of four weeks was used to provide additional information on the regeneration of nerve tissue after cryosurgery.

Materials and Methods

General anaesthesia was administered via a closed system of fluothane, nitrous oxide and oxygen, with the appropriate mask. The skin over the left hind leg was shaved and disinfected in preparation for surgery. Over the mid-thigh region an incision 3 cm long was made, the muscles were exposed and separated bluntly. The sciatic nerve was exposed and 5 ml of 4 per cent gluteraldehyde in cacodylate buffer pH 7·3 was flushed over the exposed nerve in situ. After 5 minutes, a 1 cm length of the nerve was excised and placed in 25 ml of fresh fixative. The animal was then sacrificed with 1 cc Expiral (intraperitoneal).

After fixation for 15 minutes, the nerve trunk was

cut longitudinally, with a sharp razor into two equal parts. One half of the nerve was processed for electron microscopy using the same method as in Chapter 2. The second half was prepared for light microscopy by washing in distilled water for 15 minutes and post fixation in 10 per cent buffered formalin for three days at room temperature. The tissue was processed by Page's double embedding method. Longitudinal and transverse sections were cut and stained with the following stains:-

i) Haematoxylin and Eosin; to show the nuclear components of the nerve trunk and axoplasm.

ii) Silver impregnation (Palmagren's method) to demonstrate the myelinated axons.

iii) Loyez's haematoxylin method for myelin.

iv) Mason's trichrome stain for connective tissue.

v) Luxol fast blue method for myelin.

The sections of the plastic embedded material prepared for electron microscopy were stained with toluidine blue to show myelinated and non-myelinated axons. The different stains and fixation techniques were first tried on normal material to test their efficiency and suitability for the experimental study.

Experimental technique

The sciatic nerve in eight adult Guinea pigs (weighing between 750 and 850 gm) was exposed as before. Two thermocouple needles were inserted, one directly underneath the nerve trunk and the other 1 cm away. A 1 mm cryoprobe of a C.E.8 cryosurgical machine was applied directly over

the nerve. Liquid nitrogen was used as the refrigerant and when the ice ball had grown to an area of 1 cm and the indicators showed a temperature of $-30^{o}C$ the cryogenic treatment was halted. The nerve was allowed to thaw slowly, the muscles were replaced and the skin was closed and sutured by a simple interrupted method using a nylon thread. After recovery the animals showed paralysis of the treated limb.

The animals were left 20 minutes; two hours; one, two and three weeks; 26 days and 28 days after the operation. By two weeks the remaining animals showed inflammation in the toes and in one case the animal had to be sacrificed at 26 days post operatively.

Each biopsy was taken under general anaesthesia as previously described. In order to mark the nerve trunk for easy identification, the proximal end was cut in a straight line at right angles to the long axis and the distal portion was cut obliquely. The animals were sacrificed after removal of the portion of the nerve. The biopsies were treated in the same was as the normal tissue.

Observations

a) The normal nerve trunk

In transverse section the sciatic nerve consisted of many rounded fascicles of different sizes (fig. 5.1). These fascicles were surrounded by a thick connective tissue layer (fig. 5.1, 5.3, 5.4) which consisted mainly of bundles

of collagen and a few elastic fibres. Most of the connective tissue fibres were parallel to the long axis of the nerve trunk (fig. 5.3, 5.5). This outer connective tissue component is called the epineurium (fig. 5.1, 5.2). The epineurium contained fibroblasts and blood vessels (fig. 5.3). The epineurium was surrounded by adipose tissue (fig. 5.4). The connective tissue elements arising from the epineurium extended between and around the nerve bundles (fig. 5.1, 5.2). This layer of connective tissue is called the perineurium. Within each nerve bundle, the individual nerve fibres were surrounded by a thin, delicate layer of collagen fibre bundles, called the endoneurium (fig. 5.6,)
The endoneurium was attached to the neurolemma membrane (fig. 5.6,)

The sciatic nerve of the Guinea pig consisted of many myelinated nerve axons and only a few non-myelinated axons (fig.5.7) Both the non-myelinated and myelinated nerves were orientated parallel to each other (fig. 5.5) although the conglomeration of nerve fibres often followed an undulating course. The myelinated nerveswere of different diameters ranging from the large at 5·6 µm to the small of 131·3 nm diameter (fig. 5.7). The myelinated nerves contained a central round or oval axon surrounded by a myelin sheath (fig. 5.7). The Schwann cell nucleus was located at the periphery of the myelin sheath (fig. 5.6). There were a few fibroblasts present amongst the collagen fibres of the endoneural tube (fig. 5.6). The few non-myelinated axons were aggregated in clusters between the myelinated nerves, the non-myelinated nerves

were associated with one or two Schwann cell nuclei (fig. 5.7). The non-myelinated nerves were also surrounded by a collagenous endoneural tube (fig. 5.7).

In the longitudinal section (fig. 5.8), the myelinated nerves extended for the whole length of the biopsy. The myelin was constricted in places and the continuity was interrupted, forming a segmented pattern (fig. 5.8). At the point of discontinuity the myelin was absent from the axon (fig. 5.8); these areas are called the nodes of Ranvier. In the inter-nodal segments the myelin was interrupted by oblique fissures which are called the Schmidt Lanterman incisures (fig. 5.9).

At high power, a section stained with Masson's trichrome, showed the fine endoneurium associated with each nerve fibre (fig. 5.10). The nerve fibres (fig. 5.10) consisted of a dark central core surrounded by a pale zone (fig. 5.10). The dark central core was the axon of the nerve fibre. The axon contained the axoplasm which was continuous through the nodes of Ranvier and Schmidt Lanterman incisures (fig. 5.8). The pale zone surrounding the axon contained fine reticulate structures which consisted of neurokeratin (fig. 5.10). The neurokeratin was found in the myelin sheath but was obscured by myelin staining agents (fig. 5.8, 5.9).

b) Twenty minutes after treatment

The general architecture of the nerve fascicles was virtually intact (fig. 5.11). The nerve bundles were surrounded by layers of perineural connective tissue and

were well circumscribed, round compartments. The fibroblasts of the epineurium showed early degenerative changes (fig. 5.12), most of the cells were still spindle shaped but the nuclei were pyknotic.

The blood vessels had become much more conspicuous (fig. 5.11, 5.12) as they were engorged with erythrocytes. The walls of some blood vessels had ruptured (fig. 5.12) and the endothelial cells contained vacuolated granular cytoplasm and pyknotic nuclei (fig. 5.12). The connective tissue surrounding these vessels was virtually intact. The inter fascicular fine blood capillaries showed early degenerative changes in the endothelial cells (fig. 5.14) but most of then were still intact and filled with fine granular oedematous fluid or microthrombi (fig. 5.11, 5.14).

The endothelial collagen fibres (fig. 5.13) were still almost intact, surrounding the nerve fibres but around some of the larger nerve axons the endoneural tube was ruptured and distorted (fig. 5.13). In other places the collagen fibres seemed to have peeled away from the surface of the nerve (fig. 5.13,)

The longitudinal sections stained with toluidine blue showed nerve bundles which were irregular and wavey in appearance (fig. 5.15). The myelin layer had a mottled granular appearance compared to the homogeneous dense normal myelin (fig. 5.7, 5.9). The myelin of large nerve axons showed more distortion than that of the small diameter axons (fig. 5.16). Schmidt Lanterman

incisures were more frequent along the length of the axon and were open (fig. 5.15). The nodes of Ranvier were wide and distorted and the myelin had retracted (fig. 5.15.) leaving a longer length of naked axon.

The Schwann cells within the internodal spaces showed early degenerative changes (fig. 5.13, 5.14) as the nuclei were pyknotic.

At low power the nerve axons had swollen and lost their discrete normal structure (fig. 5.11). In sections stained with haematoxylin and eosin the dark cores of the axons were swollen (fig. 5.13) and often displaced from their central position. The pale zone surrounding the dense central core of the normal nerve axon (fig. 5.9) had become much denser (fig. 5.13, 5.14) and the network of fine filaments had become more conspicuous and condensed (fig. 5.13).

c) Two hours after treatment

By two hours post operatively the evidence of injury to the sciatic nerve had greatly increased (fig. 5.17), particularly in the centre of the lesion. The nerve bundles within the fascicles had separated from the perineurium (fig. 5.17, 5.19). There was even some separation of individual axons within the fascicles. One conspicuous feature of the biopsy at two hours after treatment was the large number of leucocytes in the epineurium. The large blood vessels were congested with erythrocytes and leucocytes (fig. 5.18, 5.19). The endothelial cells of the blood vessels were distorted and in some the walls had ruptured (fig. 5.19).

The endothelial collagen fibres surrounding the nerve axon were irregular, distorted and often were separated from their nerve fibres (fig. 5.20). Intra-fascicular blood capillaries were congested with red blood cells and their endothelial cells showed degenerative changes (fig. 5.21). There were a few red blood cells in the oedematous fluid found between the nerve fibres (fig. 5.21).

In longitudinal section, the large myelinated nerve fibres showed the effects of injury as distorted, wavey nerve fibres with swollen myelin layers (fig. 5.19, 5.22). With Loyez's stain the myelin had a honeycomb appearance (fig. 5.22). A network of dense material surrounded by translucent "empty areas" (fig. 5.22) was found peripheral to the axon. In sections stained with osmium tetroxide and toluidine blue (fig. 5.23a) the myelin had a beaded appearance.

The myelin was particularly distorted at the nodes of Ranvier, which had resulted in an increase in length of exposed, naked axon (fig. 5.23a). The axons had contracted and the Schmidt Lanterman incisures had been brought closer together (fig. 5.23). Schwann cell nuclei were flattened and pyknotic (fig. 5.21). The fine myelinated axons were less distorted than the larger ones. Due to the extreme distortion of the myelinated nerves, it was difficult to distinguish the non-myelinated nerve axons.

In haematoxylin and eosin stained, transverse sections the dark central cores of the axon (fig. 5.20) were either circular or crescent shaped and the majority were displaced

from the normal central position (fig. 5.20). The peripheral clear zones were either devoid of any structure or contained a variable amount of reticular material (fig. 5.20).

The severity of the degenerative changes decreased towards both ends of the nerve trunk biopsy. The distal and proximal ends showed similar changes.

d) Twenty four hours after treatment

Sections taken from biopsies twenty four hours after treatment showed an increased distortion and further degenerative changes. The major changes were seen in the central region and decreased towards the proximal and distal stumps. The distal stump showed more degenerative changes than the proximal part of the nerve trunk.

In the central region the epineural connective tissue still surrounded the nerve fascicles (fig. 5.24) but the connective tissue bundles were divided by large spaces. The epineurium contained acidophilic, thick homogeneous masses, irregular fat droplets, red blood cells and degenerated fibroblasts (fig. 5.25, 5.26). The blood vessels of the epineurium contained degenerate endothelial cells and were packed with erythrocytes (fig. 5.26). Frequently the walls of the blood vessels were ruptured (fig. 5.27). The epineurium and perineurium contained many lymphocytes (fig. 5.25, 5.26). The perineural fibroblasts were degenerate (fig. 5.26) some were swollen with pyknotic nuclei and vacular cytoplasm, whilst others were contracted, thin and elongated with a highly basophilic

nucleolus (fig. 5.27). The endoneural connective tissue between the nerve fibres showed disruption (fig. 5.30) and discontinuities. The disruption and disorganisation of the endoneural collagen fibrils was more obvious in the larger nerve fascicles (fig. 5.25).

Blood capillaries between the endoneural connective tissue contained microthrombi filling the lumen of the vessels, the endothelial cells were highly degenerate and necrotic (fig. 5.24).

The greatest degree of separation of the nerve fibres within the bundles was seen in the largest fascicles (fig. 5.25). In the smaller fascicles the nerve fibres were much less separated (fig. 5.25). Most of the discrete separated fibres were still surrounded by endoneural tubes, the spaces between the separated nerves contained a few collagen fibres (fig. 5.28). The nerve fibres in the centre of the lesion were highly distorted and degenerate (fig. 5.29). The myelin was either degenerate, fractured or had a beaded appearance (fig. 5.28). At the nodes of Ranvier the myelin had retracted considerably, leaving exposed axons (fig. 5.28). The Schmidt Lanterman incisures were injured and closed (fig. 5.28).

By 24 hours post-operatively both large and small diameter nerve fibres showed definite degeneration. In transverse sections, the axons of myelinated nerves were contracted and darkly stained with silver (fig. 5.29). The axons had irregular outlines and were frequently displaced from the normal central position (fig. 5.29).

The injured axons were surrounded by degenerate myelin (fig. 5.30) which had a granular structure in sections stained with haematoxylin and eosin. Schwann cells were also degenerate and the nuclei were pyknotic or fractured (fig. 5.29).

The non-myelinated nerve axons were difficult to distinguish.

In the proximal part of the nerve trunk the degenerative changes were not as marked as in the centre of the lesion (fig. 5.31). The fascicular arrangement of the nerve bundle was still intact, the large blood vessels in the epineurium were congested with erythrocytes and leucocytes.

The large diameter, myelinated nerves showed more early degenerative changes than the smaller ones (fig. 5.29). The myelin was irregular and had retracted leaving elongated nodes of Ranvier.

The distal region of the nerve trunk showed more signs of injury than the proximal region but not such extensive damage as in the centre of the lesion.

e) <u>One week after treatment</u>

One week after treatment, the nerve trunk showed three different areas, i) the proximal region, ii) the central region and iii) the distal region. There was no definite junction between the regions as the appearance merged gradually from zone to zone. The proximal region showed some degeneration and also signs of regeneration. The central region contained only necrotic nerve debris and the distal region contained nerve fibres still at various

stages of degeneration.

i) The proximal region

The most significant observation one week after treatment was the appearance of regenerated nerve fibres (fig. 5.32). In longitudinal section the regenerating nerve fibres were fine myelin threads situated within the old endoneural tubes (fig. 5.32). Many of the endoneural tubes contained no recognisable structures (fig. 5.33), whilst others contained Schwann cell nuclei (fig. 5.33). There were more Schwann cell nuclei in the normal nerve bundles (fig. 5.34). Several Schwann cell nuclei were lined up within the endoneural tubes (fig. 5.35). In transverse section the small regenerated nerve fibres and their myelin sheaths were easily recognised (fig. 5.36).

Phagocytes and fibroblasts were present in the epineurium (fig. 5.37). There was an increase in the numbers of fibroblasts and the amount of collagen in the endoneural connective tissue (fig. 5.37). The endoneural tubes were still intact surrounding the nerve fibres (fig. 5.33). The connective tissue components of the nerve trunk contained numerous fine blood capillaries (fig. 5.37).

ii) Central region

The central region showed extensive nerve degeneration although the perineurium and epineurium had retained their integrity. The perineurium and endoneurium contained many phagocytes and microthrombi (fig. 5.38). The fibroblasts in the epineurium were active (fig. 5.38) and some of the blood vessels were normal (fig. 5.38). The nerve bundles

also contained many congested blood capillaries and phagocytes (fig. 5.39). The blood capillaries within the nerve bundles were more numerous one week after treatment than at 24 hours. The myelinated nerves were totally distorted and degenerate (fig. 5.40). The endoneural tubes contained only granular necrotic debris (fig. 5.40).

iii) Distal region

There was more evidence of injury to the nerve trunk in the distal region than in the proximal region and less than in the central zone (fig. 5.41). There were less congested blood capillaries in the nerve bundles (fig. 5.41) than in the central region (fig. 5.38). The endoneural tubes were distorted (fig. 5.42) and contained necrotic debris and the occasional Schwall cell nucleus (fig. 5.42). The nerve fibres, and in particular the myelin, were distorted and abnormal (fig. 5.43). Occasionally in the small diameter fibres the Schmidt Lanterman incisures had escaped injury (fig. 5.43), but the nodes of Ranvier were disrupted.

f) Two weeks after treatment

i) Proximal region

The myelinated nerve fibres in the proximal region two weeks after treatment were either degenerate or contained fine branched filaments (fig. 5.44). The fine branches were surrounded by a thin layer of myelin (fig. 5.44). The fine nerve fibre branches had passed through the nerotic nerve debris (fig. 544). In the same areas as the branched fine nerve fibres were many new Schwann cell nuclei (fig. 5.45).

Frequently the Schwann cell nuclei were lined up in groups, juxaposed to the endoneural tube (fig. 5.45). Many other nerve fibres were disrupted and contained necrotic debris (fig. 5.48). Structures thought to be regenerated non-myelinated nerve axons were well circumscribed and basophilic (fig. 5.48).

Most of the endoneural tubes were intact (fig. 5.45) and interspersed with fibroblasts and macrophages (fig. 5.46). At two weeks post operatively there were more fibroblasts in the epineural and perineural connective tissue than at one week after treatment (fig. 5.47). The intra-fascicule blood capillaries had a normal appearance and were fully regenerated (fig. 5.46).

ii) Central region

The myelinated nerves were degenerate and fragmented (fig. 5.49). The normal structure of the nerve fibres was no longer discernible. A conspicuous feature of the nerve bundles were the large numbers of new Schwann cell nuclei (fig. 5.49). The Schwann cell nuclei were often found within the remaining endoneural tube (fig. 5.50). Frequently, the endoneural tubes contained only scattered floccular material (fig. 5.50). Occasionally regenerated nerve ends were observed, which were surrounded by Schwann cells (fig. 5.51).

The non-myelinated nerves were more numerous and easier to distinguish (fig. 5.51) than in previous biopsies.

The connective tissue component of this biopsy showed an increased thickness of epineurium (fig. 5.53). Several

macrophages were present in the vicinity of the blood vessels. The blood vessels in the epineurium were normal and the blood capillaries were more numerous (fig. 5.58). Fibroblasts were common throughout the connective tissue (fig. 5.50, 5.53). The endoneural connective tissue showed signs of recovery as there was less separation from the nerve fibres (fig. 5.50). The regenerating epineural connective tissue had replaced the adipose tissue normally surrounding the nerve trunk (fig. 5.54).

iii) Distal region

The nerve trunk was still continuous and the degenerative changes had decreased distally. There were no regenerating, fine nerve fibres in the distal region (fig. 5.55) only damaged and degenerate nerve fibres. Many Schwann nuclei were degenerate and pyknotic (fig. 5.55). Non-myelinated nerves could be distinguished but they were not as numerous as in the central zone.

The connective tissue elements of the nerve trunk had the same appearance as in the central region.

g) Three weeks after treatment

i) Proximal region

The appearance of the myelinated nerves was very similar to the biopsy taken two weeks post treatment. Fine newly regenerated nerve fibres were present amongst the degenerate remnants of the injured fibres (fig. 5.56). The regenerated nerve fibres showed no nodes of Ranvier or Schmidt Lanterman incisures (fig. 5.56) but were branched. Whilst there was little change in the myelinated nerve fibres

the non-myelinated fibres showed a distinct increase in numbers (fig. 5.57). Associated with the non-myelinated nerves were Schwann cells (fig. 5.57).

There was a slight increase in the thickness of the endoneural collagen fibres (fig. 5.57). The epineurium and perineurium showed little change from the two week biopsy.

ii) Central zone

The regenerated nerves had a small diameter (fig. 5.58, 5.60) and were situated within old endoneural tubes. The degenerate, injured nerve fibres were more numerous than the regenerated nerve fibres (fig. 5.58). The damaged fibres contained necrotic debris and coagulated clumps of myelin (fig. 5.58). No Schmidt Lanterman incisures or nodes of Ranvier were seen in either the regenerated or the injured nerve fibres (fig. 5.58).

As in the proximal region there was an increase in the numbers of non-myelinated nerve fibres from two to three weeks post operatively (fig. 5.59). Schwann cell nuclei were closely associated with the groups of non-myelinated fibres (fig. 5.59).

The endoneurium was cohesive and thicker (fig. 5.60); it contained many fibroblasts and macrophages. The endoneural tubes contained scattered, floccular debris and the small regenerated nerve fibres (fig. 5.60). The regenerated nerve axon was situated at the periphery of the endoneural tube (fig. 5.60). The blood capillaries in the endoneurium had a normal structure (fig. 5.61).

The perineurium at three weeks was little changed from the biopsy taken two weeks after treatment. However, the epineurium at three weeks had greatly increased in thickness (fig. 5.62) and had fused with the epimysium of the juxtaposed muscle. There was no adipose tissue in the epineurium (fig. 5.62). The juxtaposed muscle, which had also been damaged during the treatment, showed signs of repair and regeneration (fig. 5.62).

iii) Distal region

At three weeks post operatively there were more regenerated nerve fibres in the distal stump than in the distal region two weeks after treatment (fig. 5.63). The majority of the regenerated nerves were not myelinated (fig. 5.63).

There were no conspicuous changes in the connective tissue components, three weeks after treatment.

h) Four weeks after treatment

i) Proximal region

By four weeks most of the necrotic debris had been removed (fig. 5.64) and within some of the nerve fascicules the compartmental structure was in process of being established (fig. 5.64).

There was an increase in the numbers of regenerated nerve fibres (fig. 5.64) and often these were branched (fig.5.65). A significant observation was the presence of nodes of Ranvier and Schmidt Lanterman incisures in the regenerated nerve fibres (fig. 5.65).

ii) Central zone

In the central zone there were more areas of necrotic

debris than in the proximal zone four weeks after treatment (fig. 5.66). The regenerated nerve axons were surrounded by a thin layer of myelin but showed no Schmidt Lanterman incisures or nodes of Ranvier (fig. 5.66, 5.67). The non-myelinated nerve fibres were inconspicuous. The Schwann cell nuclei were numerous and situated at the periphery of the endoneural tube (fig. 5.68). The endoneurium contained many fibroblasts (fig. 5.68).

The thick perineurium was intimately associated with the perimysium of the neighbouring muscle (fig. 5.69).

iii) Distal region

Very few of the regenerated nerve fibres were myelinated (fig. 5.70). The distal region contained more necrotic debris than the proximal and central regions (fig. 5.70). The nerve fibres were aggregated into compartments within the fascicles (fig. 5.70).

The cellular elements in the epineurium and perineurium had increased (fig. 5.71), otherwise there was little change.

Discussion

Degeneration of the nerve fibres progressed rapidly for the first 24 hours after cryosurgery. Regeneration and repair were first seen one week after treatment and were still in progress at four weeks.

The process of axon disintegration is called Wallerian degeneration, in recognition of Waller (1850) who first described the changes visible in the light microscope. These observations have since been confirmed on many

occasions. The significant feature of Wallerian degeneration is that the primary lesion affects the axon with only secondary involvement of the Schwann cell and loss of myelin. This is in contrast to segmental demyelination in which the lesion occurs exclusively in the Schwann cell (Allt, 1976). Allt (1976) suggested that the term Wallerian degeneration should only be used to describe changes distal to a cut or crush injury. However, the term is now in common usage to describe this type of degeneration regardless of the nature of the injury. Wallerian degeneration describes the injury distal to the lesion and is frequently accompanied by retrograde degeneration proximal to the lesion.

Seddon (1943) was one of the first workers to produce a classification of nerve injury after mechanical trauma. Seddon proposed three categories of nerve injuries with a clinical bias. A more recent classification was proposed by Sunderland (1968) based on the extent of damage to the internal anatomy of the nerve trunk. The classification related to lesions producing loss of function only. Five degrees of injury (Sunderland, 1968) may be defined which involve successive and progressive loss of

1. conduction of the axons
2. continuity of the axons without breaching the endoneurium
3. continuity of nerve fibres with disruption of the endoneurium
4. the perineurium and fascicular structure
5. continuity of the nerve trunk.

Cryosurgery produced a second degree of injury in the majority of nerve fibres. Second degree injury (Sunderland, 1968) involves severing of the axon or the axonal mechanisms are so disorganised that the axon fails to survive below the level of the injury and for a variable but short distance proximal to it. The general structure of the endoneurium and remaining structures comprising the nerve trunk are mostly preserved (Sunderland, 1968).

A third degree injury (Sunderland, 1968) results from more severe trauma which, in addition to causing axonal disintegration, disorganises the internal structure of the fascicules. The essential change is a disorganisation of the internal structure of the bundles in which endoneural tube continuity is destroyed.

The cryosurgical treatment did not destroy the endoneural tubes and was essentially a second degree injury. This confirms the observations of Carter et al. (1972). The significance of the preservation of the endoneural tubes was seen in regeneration when the intact portion of the axon started to grow distally, confined to the endoneural tube it originally occupied.

The first stage of Wallerian degeneration after a cut or crush injury is a change in shape of the axon and retraction of the myelin sheath at the nodes of Ranvier (Allt, 1976). The retraction of the myelin sheath creates increased nodal gaps. Myelin sheath retraction at the nodes of Ranvier were seen in the present study 20 minutes after treatment. Williams and Hall (1971)

observed nodal widening of a small axon within two minutes of a crush injury. In some of the larger fibres the nodal widening did not occur for 60 minutes (Williams and Hall, 1971).

The changes in the Schmidt Lanterman incisures observed in the present study have also been reported after crush injury (Webster, 1965). Webster (1965) described the increase in the number of Schmidt Lanterman incisures and proposed that this occurred as a prelude to myelin breakdown. It is thought that an essentially degradative process was most unlikely to produce de novo such complex and orderly structures as Schmidt Lanterman incisures. Williams and Hall (1971a) consider it more likely that the increase in numbers was due to a sudden dilatation of previously closed incisures. It is proposed that the contraction of the myelin sheath or lengthening of the nodal gap would bring the incisures closer together and therefore bring more incisures into the field of view. The dilatation of most incisures followed by a progressive collapse led Williams and Hall (1971 a and b) to suggest that they act as foci for the disruption of the myelin sheath.

The precise mechanism of injury by cryosurgery is only speculative. Lenz and Noack (1974) showed that ice crystal formation, osmotic changes and enzymic changes were all involved in nerve destruction. Blood vessel stasis and ischemia have also been suggested as factors important to nerve fibre disruption during a freeze thaw cycle (Carter, Lee, Gill and Johnston, 1972).

Whittaker (1974) reported that ischemia could play only a very small role in the early stages of disruption, the observations in the present study are consistent with Whittaker's (1974) observations.

Lipoprotein molecules have been shown to be denatured by very low temperatures (Benta and Code, 1972). The myelin sheath and the axolemma are both made up of lipoproteins (Lovelock. 1957) and after cut or crush injury the loss of lipids is considered secondary to the breakdown of myelin protein by proteolytic enzymes (Hallpike, 1972). Acid and neutral protease activity has been detected as early as 24 hours after cut or crush injury (Allt, 1976) with a two or three fold increase in the first week. The additional effect of denaturation by low temperature probably accounts for the increased intensity and speed of degeneration observed in the myelin sheath after cryosurgery. Indeed, the low temperature degradation of myelin could well be the primary cause of myelin disruption. The enzymes responsible for myelin degeneration are thought to be derived from the axons, Schwann cells and macrophages (Allt, 1976).

The effect of cryosurgery is more drastic on tissue elements which contain a high proportion of water. The endoneural collagen fibres and their matrix have only a low water content compared to the axoplasm. Consequently the endoneurium was preserved after the cryosurgery but the axons were highly disrupted particularly in the central zone. In order to create the same degree of axon injury

after mechanical trauma the connective tissue elements of the nerve trunk would have shown excessive damage.

The central zone showed well defined and extensive injury to the nerve trunk. This is thought to be due to four factors which are as follows:-

i) the low temperature achieved

ii) the rate of cooling and thawing

iii) a physical bond between the cryoprobe and nerve trunk

iv) the pressure of the cryoprobe.

As might be expected, the lower the temperature the greater the extent of injury; a temperature of $-20^{o}C$ will cause more injury than a temperature of $-10^{o}C$ (Caster, Davidson, Rand and Funkalsruel, 1971). The tissue temperature directly underneath the cryoprobe would have been greater and been achieved faster than at the periphery.

During freezing the area directly under the probe would have frozen first and thawed last creating different cooling and thawing rates than at the periphery. The rate of cooling and rewarming has been shown to have an influence on the degree of cell injury and their subsequent survival (Jagodzinski, 1966).

It is difficult to assess the possible physical injury to the nerve caused by bonding with the cryoprobe. Slight increments in pressure during the manipulation of the cryoprobe during freezing might also have had an effect.

Pressure application of the cryoprobe tip associated with freezing also has an effect on the extent of damage

to the nerve (Leopard et al., 1974).

The presence of oedematous fluid was associated with distortion of the connective tissue. However, by 24 hours there was a significant reduction in the amount of oedematous fluid. The oedematous fluid was probably derived from injured blood vessels. Increased permeability of endoneural blood vessels after crush and ligation has been shown by Olsson (1966) and by Mellich and Cavanagh (1967, 1968). The reduction of oedema by 24 hours could have been achieved by the action of the perineurium. The perineurium is recognised as a metabolically active diffusion barrier (Shantha and Bourne, 1968) and contributes to the maintenance of the composition of the endoneural matrix.

By one week after treatment the cryolesion showed three distinct zones; a proximal region showing the least injury and most regeneration; a central zone which had been established at the early stages and a distal zone which was still showing early stages of Wallerian degeneration. At one week there was an increase in numbers of phagocytes and macrophages which was thought to be associated with the establishment of tiny blood vessels. The phagocytes and macrophages showed no evidence of penetration of the endondural tubes.

The appearance of active fibroblasts between the collagen bundles one week after treatment indicated that the repair process had commenced. The fibroblasts could have migrated from the proximal region of the injured nerve

or possibly from the connective tissue surrounding the nerve trunk.

By the time the myelin had degenerated to form ellipsoids there was a proliferation of Schwann cells. There were more Schwann cells in the proximal zone than the central and distal zones; which may indicate the origin of the new Schwann cells. The exact origin and extent of migration of new Schwann cells is still largely unknown. Causey (1960) suggested that the Schwann cells proliferated and bridged a traumatised area from both sides of the injury. The extent of the injury may well be a factor in determining the origin of the new Schwann cells. The new Schwann cells arranged themselves into non-branching rows called the bands of Bunger. The presence of intact endoneural tubes appeared to be important in the orientation and organisation of the Schwann cells.

Abercrombie and Johnson (1946a) postulated that the increase was related to a chemical stimulus created by the degenerate nerve fibres. It seems more likely that the proliferation and migration of Schwann cells was related to the presence or absence of myelin. Both in vivo and in vitro studies have shown a sharp rise in the number of Schwann cells rising to a peak at 19 - 25 days followed by an equally sharp decline (Abercrombie and Johnson, 1942, 1946; Thomas, 1948; Abercrombie, Evans and Murray, 1959). Lubinska (1961) observed the phases of migratory and proliferative activity occurring either before the formation or after the destruction of myelin. The general maintenance

and normal functioning of the axon is much more compatible with a sedentary and non-dividing Schwann cell. The Schwann cells and their intact myelin may well inhibit division and migration. When the myelin is destroyed or not present and the inhibiting factor is removed, then Schwann cell division and migration can occur. This type of reaction has been clearly demonstrated in epithelial tissue (Bullough and Lawrence, 1964).

There were larger numbers of new Schwann cells in the large myelinated nerves than in the smaller ones. This was observed by Thomas (1948) and Joseph (1947, 1950). The largely non-myelinated nerves e.g. rabbit abdominal vagus produce significantly fewer new Schwann cells than a largely myelinated nerve (Abercrombie, Evans and Murray, 1959). The reason for the much more extensive proliferation of Schwann cells in the myelinated nerve compared to the non-myelinated nerve has not yet been elucidated. Although this discrepancy could be explained by the observations in the next chapter which show that the non-myelinated nerves suffer much less injury than the myelinated ones.

The first signs of regeneration were seen in the proximal zone as fine tiny sprouts. These branching sprouts were within the endoneural tubes. The regenerated axons passed through the necrotic debris indicating that axon growth and regeneration was not related to the clearance of the endoneural tubes.

The rate of regeneration of axons has been shown to be

related to the species and the type of nerve lesion (Guth, 1956; Allt, 1976). The number of nerve fibres after a crush injury were the same in the proximal and distal stumps (Allt, 1976). After nerve section the numbers of new axons in the distal stump were considerably reduced (Allt, 1976). After freezing, Mira (1979) showed an increase in numbers of regenerated nerves in the distal stump but the diameter of the axons was reduced.

The numbers of regenerated nerve axons increased in all three zones during the period of this study. The increase in regenerated axons was related to the decrease in necrotic debris. The removal of the necrotic debris will be fully discussed in the next chapter.

The adipose tissue in the epineurium did not regenerate and the reason for this is unclear. The epineurium increased in thickness with time and eventually fused with the surrounding muscle tissue. It was not clear whether the scar tissue of the neighbouring muscle or the nerve had proliferated to form the adhesion. As there was no histological differentiation into two distinct areas within the scar it is most probable that both injured areas were responsible for the formation of the adhesion. The absence of adipose tissue to protect the regenerating nerve trunk might have stimulated the fusion of the two tissues.

The regeneration of the nerve fibres will be more fully discussed in the next chapter. The ultrastructural observations add much to our understanding of the regenerative processes.

CHAPTER 6

ELECTRON MICROSCOPE OBSERVATIONS ON THE EFFECTS OF CRYOSURGERY ON THE SCIATIC NERVE OF THE GUINEA PIG

Introduction

Nerve fibres degenerate at different rates, the rate depending on such factors as the experimental animal used, the size and degree of myelination of the fibre and the type of injury inflicted on the nerve. Our knowledge of degenerative processes in nerves is incomplete and in particular after cryosurgery.

This chapter is concerned with the electron microscopy of the degenerative changes that took place within twenty four hours, of cryosurgical application to the sciatic nerve of the Guinea pig. A short report of the normal ultrastructure of the sciatic nerve of the Guinea pig is included.

Materials and Methods

i) Surgical procedures. See chapter 5, materials and methods section i).

ii) Electron microscopy. See chapter 3, materials and methods section iii).

Observations

A Normal

The sciatic nerve consisted of bundles of myelinated and non-myelinated nerve fibres. The myelinated nerves were more numerous than the non-myelinated nerve fibres (fig. 6.1). The bundles of nerve fibres were surrounded by connective tissue. The outermost layer of connective tissue was the epineurium which was composed of collagen fibres orientated parallel to the longitudinal axis of the nerve trunk (fig. 6.1, 6.2). Between the collagen fibres were several fibroblasts and blood vessels (fig. 6.2). From the epineurium a layer of collagen fibres extended around the nerve fasciculi called the perineurium (fig. 6.3). Interspersed between the bundles of collagen fibres of the perineurium were flat, elongated fibroblasts (fig. 6.3). From the perineurium collagen fibres extended to surround the individual nerve fibres (fig. 6.1). The collagen fibres and accompanying fibroblasts (fig. 6.4) surrounding the nerve fibres are called the endoneurium.

Axons in the peripheral nervous system were surrounded by sheet like processes from the Schwann cells. The cytoplasmic encasement of the axon could be simple (non-myelinated) (fig. 6.1) or could involve a complicated, multilayered enclosure when the axon is said to be myelinated (fig. 6.1, 6.4).

i) The myelinated nerve fibres

The outermost part of the Schwann cell was the neurolemina (fig. 6.5) and it contained mitochondria, Golgi bodies, ribosomes and a nucleus (fig. 6.5, 6.6). The Schwann cell nuclei was oval and had a peripheral position in the cell (fig. 6.6). The outer surface of the Schwann cell was surrounded by a thin external basal lamina (fig. 6.5). The basal lamina was separated from the cell membrane of the Schwann cell by a zone containing granular material 1 nm thick (fig. 6.5). The innermost portion of the Schwann cell cytoplasm was wrapped around the axon forming the myelin sheath (fig. 6.5). During the wrapping process the inner leaflets of the trilemmelar cell membranes had fused (fig. 6.5) to give the typical pattern. The average distance between inner fused leaflets was 1·5 nm.

There were, at regular intervals, funnel-shaped, oblique clefts in the myelin sheaths (fig. 6.7) called the Schmidt Lantermann incisures. The Schwann cell cytoplasm was retained in the region of the incisure (fig. 6.7) but the fusion of the inner leaflets of the cell membrane were opened (fig. 6.7).

Each Schwann cell covered a segment of the axon and the region where adjoining Schwann cells juxtaposed is called the node of Ranvier (fig. 6.8). At the nodes of Ranvier (fig. 6.8) the condensed cytoplasmic laminae of the myelin sheath had opened to enclose pockets of paranodal cytoplasm.

ii) Non-myelinated nerves

In non-myelinated nerves several axons invaginated the cytoplasm of one Schwann cell (fig. 6.9). The cytoplasm enclosed each axon by a simple folding and overlapping of the cytoplasmic sheet (fig. 6.9). The Schwann cell nuclei had a central position (fig. 6.9). The Schwann cell and enclosed axons was surrounded by a basal lamina (fig. 6.9). Non-myelinated nerves represented mostly post ganglionic nerve fibres of the autonomic nervous system.

The axons of both non-myelinated and myelinated nerves were surrounded by a smooth axolamina (fig. 6.1, 6.4, 6.9). The cytoplasm of the axon consisted of an amorphous matrix containing microtubules, neurofilaments, elongated mitochondria, varied vesicles and occasional multivesicular bodies (fig. 6.4, 6.5, 6.7, 6.9). The microtubules were cylindrical, non-branching structures (fig. 6.7) and within the lumen of the microtubules was amorphous material (fig. 6.7). The neurofilaments were thin occasionally branched units which ran parallel to the longitudinal axis of the nerve fibre (fig. 6.7). The microtubules and neurofilaments were more closely packed in the Schmidt Lanterman incisures (fig. 6.7). The mitochondria and various vesicles were most numerous at the Nodes of Ranvier (fig. 6.8).

B Twenty minutes after treatment

The biopsy taken 20 minutes after treatment showed a

highly damaged central zone. The full extent of the injury was most evident in the myelinated nerves (fig. 6.10). The myelin sheaths were swollen and disrupted and the axons had lost much of their internal structure (fig. 6.10). At the points, where the axolamina membrane had retained its contact with the myelin sheath (fig. 6.11), the myelin lamellae had a normal appearance. Where the axolamina contact with the myelin sheath had been lost the axoplasm had contracted (fig. 6.11).

The axonal cytoplasm contained degenerate mitochondria (fig. 6.11) and many fine filaments all with the same diameter (fig. 6.11). Neither microtubules nor neurofilaments could be distinguished. The axoplasm at the nodes of Ranvier were particularly vulnerable to the treatment (fig. 6.12) and the axon contained many vacuoles and vesicles which disrupted the continuity of the axon. The myelin layers enclosing the paranodal cytoplasm at the nodes of Ranvier had ruptured releasing vesicles and ribosomes into the axon (fig. 6.12). The myelin sheaths had contracted at the nodes of Ranvier exposing a larger length of internodal axon. The width of the axon at the nodes of Ranvier was constricted by an invagination of the axolamina.

The Schmidt Lanterman incisures were also very disrupted (fig. 6.13) and the myelin lamellae at the incisure had ruptured. The diameter of the axon was greatly reduced at the incisure (fig. 6.13).

The myelinated nerve Schwann cells had also suffered

injury (fig. 6.10, 6.14), the cytoplasm was condensed and floccular and contained no recognisable organelles, only a few vacuoles (fig. 6.10, 6.14). The cell membrane and basal lamina had been ruptured in places (fig. 6.10, 6.14). The Schwann cell nuclei had ruptured membranes and contained clumps of condensed chromatin and granular material (fig. 6.14).

The endoneural collagen fibres showed no structural changes after the treatment (fig. 6.10, 6.12, 6.14) although they were surrounded by oedematous fluid. The blood capillaries in the endoneurium (fig. 6.15) were relatively unaffected apart from the erythrocytes which were severely distorted.

Occasional myelinated fibres were so badly injured that the myelin sheath had become distorted and fragmented (fig. 6.16) and the axoplasm just a mass of vesicles.

The non-myelinated nerves showed fewer signs of injury (fig. 6.17). Their Schwann cell nuclei had a normal appearance (fig. 6.17). The axolemma of the non-myelinated nerves were intact (fig. 6.17). The primary indication of injury to the non-myelinated nerves was the presence of swollen mitochondria which had lost their internal structure (fig. 6.18). The non-myelinated nerves were surrounded by almost intact basal laminae (fig. 6.18).

In the proximal and distal regions the injury to the nerve fibres was slight. There were hardly discernable changes to the axoplasmic organelles and occasional focal

separation of the myelin lamellae. There was a little floccular oedematous fluid amongst the endoneural connective tissue.

C Two hours after treatment

The disruption and injury to the nerve fibres had increased by two hours post-operatively. The myelin sheaths had undergone further degeneration with changes in the lipo-protein structure (fig. 6.19). The change in the lipo-protein elements of the myelin sheath was indicated by the osmium particles which had stained the disrupted parts of the myelin sheath but not the small areas where the normal structure was preserved (fig. 6.19).

In some axons there was still some indication of the tubular and filamentous component of the axoplasm (fig. 6.19) in others the axoplasm was just a homogeneous mass (fig. 6.20). The axolemma was still intact in some nerve fibres (fig. 6.20) but showed only intermittant contact with the degenerate myelin sheath. In some nerve fibres (fig. 6.21) the degeneration of the axon and Schwann cell was extreme, leaving only a mass of vesicles, fragments of myelin and a distorted myelin sheath (fig. 6.21).

The Schwann cell cytoplasm was damaged and vacuolated (fig. 6.20) but the basal lamina was still present (fig. 6.21). The collagen fibres of the endoneurium were still present in the immediate vicinity of the nerve fibre. The normal arrangement of the collagen fibres had been disrupted (fig. 6.22) and they were surrounded by oedematous fluid containing varied cell debris (fig. 6.22).

The Schmidt Lanterman incisures and nodes of Ranvier showed little change between 20 minutes and two hours post-operatively.

Some non-myelinated nerves showed a loss of microtubules and neurofilaments (fig. 6.23) but not of multivesicle bodies or mitochondria. Other non-myelinated nerves were less injured (fig. 6.24) and contained normal microtubules and neurofilaments. The basal lamina was intact (fig. 6.23) but the Schwann cell cytoplasm consisted of a mass of lysosomal vesicles.

The majority of the nerve fibres in the regions proximal and distal to the central region showed less damage (fig. 6.25). The Schwann cells, basal lamina and the endoneurium were normal (fig. 6.25). The effects of the injury were most apparent in the Schmidt Lanterman incisures (fig. 6.26) and the myelin sheaths of some nerve fibres (fig. 6.27). Disruption of the myelin sheath was not constant along the length (fig. 6.27). The myelin sheath damage was associated with loss of mitochondrial integrity (fig. 6.27) but the microtubules and neurofilaments remained almost intact (fig. 6.27). The myelin sheath at the Schmidt Lanterman incisures was distorted but the axon was not constricted (fig. 6.26).

D Twenty four hours after treatment

By 24 hours after treatment the injury to the central region was extensive. The nerve fibres consisted of swollen, disrupted myelin sheaths and amorphous irregular

axons (fig. 6.28). The myelin sheath was frequently fragmented (fig. 6.29, 6.30) and the Schwann cell cytoplasm indistinguishable from the degenerated debris (fig. 6.29, 6.30). The basal lamina was seen around some nerve fibres (fig. 6.29) but not in others (fig. 6.30). The axoplasm was irregular, displaced and consisted of vacuoles and degenerate organelles embedded in a dense amorphous matrix (fig. 6.30).

In some nerve fibres only the basal lamina remained intact (fig. 6.31) enclosing fragments of myelin and a mass of vesicles. Endoneural collagen fibres were closely associated with nerve fibres with intact basal laminae (fig. 6.29, 6.31). The collagen fibres were not associated with nerve fibres with no basal lamina (fig. 6.30). The collagen fibres and nerve fibres were surrounded by oedematous fluid (fig. 6.30, 6.31).

By 24 hours post-operatively phagocytes had started to infiltrate the endoneurium of the central region (fig. 6.32). The oedematous fluid contained polymorphs, erythrocytes and varied cell debris (fig. 6.32). The contracted nerve fibres were separated from each other by relatively large expanses of oedematous fluid (fig. 6.33).

The distinctive appearance of the individual non-myelinated nerve axons enclosed by the Schwann cell cytoplasm had disappeared, leaving a mass of vesicles and debris enclosed by the basal lamina (fig. 6.34). Within the mass of debris some small vesicles (0·5 um) were enclosed by definate complete membranes (fig. 6.35).

These membrane bound structures were thought to be remnants of the non-myelinated axons (fig. 6.35). No normal cell organelles were discernable apart from occasional degenerate mitochondria and multivesicle bodies (fig. 6.35).

The disruption to the nerves decreased towards the proximal and distal stumps of the nerve. The effect of the treatment was greater in the distal stump than in the proximal region 24 hours post-operatively.

In the distal stump the degree of damage to the nerve fibres was dependant on the diameter of the fibres (fig. 6.36). The nerve fibres of small diameter suffered less injury than the large fibres. Some of the myelin sheaths were extremely contorted (fig. 6.36) but did not show the separation of the lamellae of the myelin sheath apparent in the large nerve fibres (fig. 6.36). The individual nerve fibres were separated from each other by oedematous fluid packed with debris (fig. 6.36).

Small nerves in the distal stump had normal axoplasm contents and axolaminae (fig. 6.37), the only sign of injury was focal separation of the myelin lamellae. The Schwann cell had a nucleus of normal appearance but vacuolization of the cytoplasm was present.

The large nerves in the distal stump (fig. 6.38) showed axons entirely filled with degenerate organelles and vesicles. There was only a small remnant of normal axoplasm (fig. 6.38). The basal laminae of the large (fig. 6.38) and small (fig. 6.37) nerve fibres were intact. The associated collagen fibres of the endoneural tube were

present and of normal appearance (fig. 6.37, 6.38).

The non-myelinated nerves in the distal stump showed early stages of degeneration (fig. 6.39). The axoplasm contained microtubules, degenerate mitochondria and lysosomal vacuoles. The associated Schwann cell cytoplasm showed evidence of more advanced disruption (fig. 6.39, 6.40). Phagocytes were present in the endoneurium (fig. 6.40).

In the proximal stump the axons were characterised by the accumulation of mitochondria and other vesicles (fig. 6.41). The axon had contracted away from the myelin (fig. 6.41) which was normal apart from occasional areas of focal separation of the lamellae. The small nerves in the proximal stump were slightly affected by the treatment. Some of the nerve fibres of large diameter were ruptured (fig. 6.42) which had allowed the accumulated debris to be released into the endoneurium.

The Schmidt Lanterman incisures in the proximal stump were injured but still recognisable (fig. 6.43). The non-myelinated nerves showed only minimal degenerative changes.

Summary of Results

1. Twenty minutes after treatment the myelin layer was disrupted and the lamellae had separated. The axons of the myelinated nerve fibres were filled with injured axoplasmic organelles. The nodes of Ranvier and Schmidt Lanterman incisures were disrupted and damaged. The Schwann cells were damaged. The non-myelinated nerves showed less injury. The basal laminae of both the myelinated and non-myelinated nerve fibres were still well preserved.

2. Two hours after treatment, the disruption and degeneration of the nerve fibres and their Schwann cells had increased. The degenerative changes were associated with an increase in oedematous fluid. The normal arrangement of the endoneural collagen fibres was disrupted. The non-myelinated nerve fibres showed less damage to the axon organelles. The proximal and distal regions of the treated nerve showed relatively less damage.

3. Twenty four hours after treatment, the extensive damage to the nerve fibres were evident. The central region showed considerable damage, in which most of the nerve fibres were fragmented but their basal laminae were still intact. Phagocytic infiltration was evident. In the proximal and distal regions the extent of the damage to the nerve depended on the size of the nerve fibre, large myelinated nerves showed more damage. The axoplasmic organelles had accumulated at the ends of the damaged nerves.

Discussion

The distribution of the nerve fibres was obvious by twenty minutes after treatment. The large myelinated nerves showed greater disruption than the small myelinated nerves and the non-myelinated nerves. The reason for this is still obscure although the same result has been observed after other types of traumatic nerve injury (Morris, Hudson and Weddell, 1972a).

Cold application is known to have a direct disruptive effect in peripheral nerve (Whittaker, 1974). Some of the reasons for nerve destruction in the early stages have been discussed in the previous chapter. It is thought that the freeze and subsequent thaw played an important role in increasing the nerve damage.

The cell membrane plays a part in stabilization of the colloidal status of the cytoplasm (Emmelot and Benedetti, 1968; De Robertez et at., 1975). It is thought that the damage to the axolemma would alter the colloidal structure of the axoplasm, causing changes in the normal axoplasmic flow. Abnormal axoplasmic flow would disrupt nerve fibre function and shape.

Microtubules and neurofilaments also participate in normal axoplasmic flow as well as the transformation of essential proteins (Landon and Susan, 1976). The progressive degeneration of the axons after treatment reflects the disintegration of the neurofilaments and microtubules. The microtubules in normal nerve fibres form the cytoskeletion (Tilney, 1968; De Robertez et al.,1975).

Low temperature produced a breakdown in the bands of microtubular sub-units (Tilney and Porter, 1967).

When the sciatic nerve of the toad was exposed to 2°C for two hours the neurofilaments and microtubules disintegrated, but then repolymerized and reappeared when the axons reached 25°C (Echandia and Piezzi, 1968). The sciatic nerve of the cat was frozen to -35°C and the microtubules and neurofilaments disintegrated but did not subsequently repolymerize (Pinner-Pool, Campbell and Decrescito, 1969). The failure to repolymerize in the Pinner-Pool et al., (1969) and present studies could be due to irreversible changes in axoplasmic pH or in the colloidal status. Some of the sub-units may have leached through the damaged axolemma. The sub-units themselves may have been irreversibly altered by the extreme cold. The present study showed the neurofilaments to be more resistant to cold than the microtubules. This is probably due to the less complicated sub-structure of the neurofilaments.

It has been proposed (Schlapfer, 1974a) that one cause of microtubule and neurofilament disintegration is an influx of calcium ions into the axon at the point of injury. By incubating segments of rat saphenous nerve in calcium-free media Schlapfer (1974a) was able to prevent break down of axonal microtubules and microfilaments; after the addition of calcium ions to the media, changes characteristic of early Wallerian degeneration were seen. Under conditions of energy deprivation, the degenerative

changes occurred more rapidly (Schlapfer, 1974b). Schlapfer (1974 a and b) proposed that a block of energy-dependent calcium-excluding mechanisms followed by an influx of calcium ions would initiate axoplasmic degeneration. The speed with which the degenerative changes commenced in the present study indicated extreme energy deprivation. Under nutrient conditions degenerative changes were delayed for 24 - 36 hours by Schapfer (1974b). The injury caused by the cold treatment to the blood vessels would cause an improved vascular and energy supply.

Several investigators (Ohmi, 1961; Lee, 1963; Holtzman and Novikoff, 1965; Donat and Wisniewski, 1973; Schlapfer, 1973) have described in cut or crush lesions early axonal changes involving focal accumulations of mitochondria, multivesicular bodies, lamellar bodies and dense bodies. This was not seen in the present study. After freezing, the degenerative changes and in particular the disintegration changes of the microtubules and neurofilaments occurred so rapidly that axoplasmic flow had ceased before the organelles could accumulate at any one point.

Whittaker (1974) had observed the disruption of the myelin sheath that was seen in the present study. The expansion of the myelin lamellae was due to alterations in the molecular association between myelin lipids and proteins (Finean, 1953). There were intact patches of myelin associated with the axolemma. The association with regions of the axolemma had preserved areas of myelin

lamellae from early damage both during the treatment and throughout the thaw. By two hours after treatment further chemical breakdown of the myelin was shown by the differential staining reaction of the osmium tetroxide. The myelin sheath was fragmented by twenty four hours after treatment which is much earlier than after other forms of trauma (O'daly and Imaeda, 1967).

The nodes of Ranvier and Schmidt Lanterman incisures were the most vulnerable regions of the axon. The intense injury at these points may have been due to the contraction of the myelin sheath. The nodes of Ranvier and the Schmidt Lanterman incisures are thought to be concerned with nutritional exchange (Glees, 1943; Gitlin and Singer, 1974) whilst maintaining nerve fibre integrity, by allowing expansion and retraction of the myelin sheath. They may well be the sites of calcium ion influx into the axon after injury.

Changes in osmolarity of the medium were responsible for the removal of some molecular components of myelin (Joy and Finean, 1963). The presence of oedematous fluid surrounding the nerve fibres, after cold injury would alter the osmolarity of the matrix and play a role in myelin degeneration.

The non-myelinated nerve fibres were less damaged than the myelinated nerves. Whittaker in 1974 also showed evidence that autonomic nerves could survive very low temperatures. The reasons for this are still obscure. However, more recent studies emphasised that the axonal

changes were essentially the same as those observed in myelinated fibres (Aguayo, Peyronnard and Bray, 1973; Matthews, 1973; Thomas and King, 1974). Thomas and King (1974) found that degeneration in non-myelinated nerves was not seen as early as in myelinated nerves. Non-myelinated axons of normal appearance were still present for five to six days after section (Thomas and King, 1974).

The greater resistance of the non-myelinated nerves indicates a more complex role for the myelin sheath in axon degeneration after injury. It also indicates that the non-myelinated nerves are more resistant to ischemia than the myelinated nerves.

The basal laminae of the severely damaged nerve fibres were intact and survived cold treatment. One suggestion for the survival of the basal laminae is an elastic property of the basal laminae which would allow them to expand during the freezing episode (Varcko and Benditt, 1972). Haftek and Thomas (1968) showed survival of the basal lamina after crushing. The basal lamina stretched to accommodate the degenerate nerve fibres.

The proximal zone showed less degeneration in general than the distal zone. The presence of intact axon organelles in many nerve fibres indicated the presence of some axoplasmic flow. The absence of intact mitochondria in the distal region showed that axoplasmic flow had ceased.

CHAPTER 7

ULTRASTRUCTURAL OBSERVATIONS ON THE REGENERATION OF NERVE FIBRES FROM ONE TO FOUR WEEKS AFTER CRYOSURGERY

Introduction

The previous chapter described the degenerative changes in the sciatic nerve of the Guinea pig after cryosurgery. The degenerative changes were rapid and extreme, particularly in the centre of the lesion. Although a study of the degenerative changes casts new information on the fundamental properties of the sciatic nerve, the reparative changes are of special interest to clinicians and surgeons. This chapter is a report of observations in the electron microscope of the regenerative changes which occurred up to four weeks after cryosurgery.

Materials and Methods

i) Surgical procedures. See chapter 6, materials and methods section i).

ii) Electron microscopy. See chapter 3, materials and methods section iii).

Observations

A One week after treatment

Although degenerative changes in the nerve were still present, one week after treatment, the first signs of nerve regeneration were present. Three zones were distinguishable, i) proximal region; ii) central region and iii) the distal region.

i) Proximal region

The general picture of the proximal stump one week after treatment was one of varied reaction by the nerve fibres to the treatment (fig. 7.1).

The small myelinated nerves had not been badly affected (fig. 7.1) but the myelin sheaths and axons of the larger nerves were very degenerate (fig. 7.1). The Schwann cells were mostly intact although there was evidence of vacuolization in the cytoplasm (fig. 7.1). The collagen fibres of the endoneural tubes were correctly orientated and distributed (fig.7.1). Macrophages and fibroblasts were numerous (fig. 7.1). The macrophages contained vacuoles filled with degenerate myelin. The fibroblasts were active with large conspicuous nuclei and rough endoplasmic reticulum cisternae packed with granular material (fig. 7.1). Some profiles of necrotic debris were found interspersed amongst the collagen bundles (fig. 7.1).

There were newly formed Schwann cells within the basal lamina of some of the nerve fibres (fig. 7.2). The new Schwann cells were most frequently found in the large nerve fibres where the damage had been most extensive (fig. 7.2). The myelin sheath had degenerated and fragments were interspersed between the new Schwann cells and the remnants of the old Schwann cells (fig. 7.2). The new Schwann cells contained prominant nuclei surrounded by cytoplasm rich in ribosomes (fig. 7.2). The cytoplasm also contained Golgi bodies and their vesicles (fig. 7.2).

One of the new Schwann cells in the micrograph showed rough endoplasmic reticulum (fig. 7.2) as well as a structure thought to be early myelin formation. This cell (fig. 7.2) was probably older than the other two and was juxtaposed to a non-myelinated nerve axon. The basal lamina of the old endoneural tube was intact (fig. 7.2) and enclosed the newly formed Schwann cells.

Many of the endoneural tubes with degenerate axons contained new axons (fig. 7.3). The new axons were small with an average diameter of 0·6 um. The new axons were surrounded by the Schwann cell (fig. 7.3) but there was no evidence of myelin formation. The myelin sheath of the original axon was highly disrupted (fig. 7.3). The old axon contained no recognisable features (fig. 7.3). There were microtubules scattered throughout the new axons (fig. 7.3). The basal lamina was intact (fig. 7.3). The Schwann cell cytoplasm contained numerous Golgi bodies and vesicles (fig. 7.3). The Schwann cell nucleus was normal and active (fig. 7.3). This was a newly formed Schwann cell and remnants of the old Schwann cell were seen at the periphery of the endoneural tube (fig. 7.3).

Where the injury to the myelin sheath and axon was extensive the Schwann cell cytoplasm was also degenerate (fig. 7.4). The Schwann cell cytoplasm contained vesicles of various sizes (fig. 7.4). The cell membrane had ruptured allowing fragments of degenerate myelin to penetrate the Schwann cell cytoplasm (fig. 7.4). The Schwann cell nucleus also showed indications of injury as

the contents had clumped together (fig. 7.4) and the nucleus had shrunk away from the nuclear membrane (fig. 7.4).

It was not possible to distinguish between non-myelinated nerves and regenerated myelinated nerves by one week after treatment (fig. 7.5). The endoneural tube containing axons and a Schwann cell in fig. 7.5 could be either a non-myelinated nerve or a myelinated nerve fibre at a more advanced stage of regeneration than in the previous micrographs.

ii) Central region

One week after treatment, the central region of the treated nerve contained many phagocytes (fig. 7.6). There were large lipid droplets in the endoneural connective tissue (fig. 7.6) and the collagen fibres were coated with osmophilic speckles (fig. 7.6). The phagocytes contained various vesicles, some of which contained fragments of myelin (fig. 7.6).

Nerve fibres of small diameter showed more disruption of their axons and myelin sheaths than the proximal zone (fig. 7.7). In some places the degenerate nerve was surrounded by a healthy active Schwann cell (fig. 7.8). The cytoplasm of the Schwann cell contained lysosomal bodies in which there were myelin-like bodies (fig. 7.8). There were numerous Golgi bodies and vesicles in the cytoplasm of the Schwann cell (fig. 7.8). The presence of a centriole indicated a recent mitosis (fig. 7.8).

In places the phagocytosis of the necrotic debris had been so efficient that the only remnant of the nerve

was a collapsed, convoluted basal lamina surrounded by collagen fibres (fig. 7.9). On other occasions the endoneural tube was complete apart from the myelin sheath and the axon (fig. 7.10). In their place was just loosely packed, floccular material (fig. 7.10). Alongside the "empty" axon was a small regenerated axon (fig. 7.10).

Some of the larger endomysial tubes were packed with new Schwann cells and regenerating axons (fig. 7.10). There was no indication of myelin formation (fig. 7.10). The newly formed axons contained mitochondria, microtubules and neurofilaments (fig. 7.10). The endoneural tubes contained structures whose identity was difficult to assess (fig. 7.10). It was not known whether these bodies were remnants of the old endoneural tube contents or regenerated structures (fig. 7.10).

The collagen fibres surrounding the endoneural tubes were sparce (fig. 7.10). There was scattered floccular material in the inter-neural tube spaces also (fig. 7.10), but very little cell debris in the areas of regeneration (fig. 7.10).

It was not possible to distinguish between the non-myelinated nerves and the regenerating nerves at this stage.

iii) Distal region

Generally the injury to the distal region was less extensive than in the central zone (fig. 7.11). The nerve fibres were separated by large spaces containing

sparce collagen fibres and extensive floccular material. In other parts of the distal region the myelin sheaths and axons had suffered greater injury (fig. 7.12).

A new axon had penetrated the degenerated axon (fig. 7.13) within the myelin sheath. The lamellae of the myelin sheath had separated but in other respects were normal (fig. 7.13). The new axon was surrounded by a neurolemma and contained mitochondria, neurofilaments and microtubules (fig. 7.13). The basal lamina of the endoneural tube was intact (fig. 7.13). The Schwann cell cytoplasm was normal in appearance (fig. 7.13). A second new axon was present within the same endoneural tube (fig. 7.13).

Other nerve fibres showed new axons peripheral to the degenerate myelin sheath (fig. 7.12).

B Two weeks after treatment

Two weeks after treatment the biopsies showed only a few changes from the appearance at one week. The most significant changes were further fragmentation and lysis of the myelin and the initiation of myelination of the regenerated nerve sprouts of the proximal zone.

i) Proximal zone

Some endoneural tubes still contained extensive cell debris (fig. 7.14). The narrow, newly regenerated axon had a tortuous course through the vesicles and other debris (fig. 7.14). The Schwann cell was peripheral with a normal nucleus (fig. 7.14). The basal lamina

of the endoneural tube was intact and closely associated with the endoneural collagen fibres (fig. 7.14). The inter-endoneural spaces contained fibroblasts, floccular ground material and membrane bound profiles of cellular debris (fig. 7.14).

The debris in other axons had largely been removed (fig. 7.15) leaving the endoneural tube packed with smaller new axons and the almost empty old axon. The macrophages were often in close proximity to the endoneural tubes (fig. 7.15).

At a more advanced stage of regeneration the Schwann cell cytoplasm had developed extensive rough endoplasmic reticulum (fig. 7.16). The cisternae of the R.E.R. were packed with amorphous material (fig. 7.16). There were several Golgi bodies with numerous associated vesicles (fig. 7.16). There was evidence of new basal lamina formation within the old one (fig. 7.16).

The small axon (0·2 um in diameter) was situated at the periphery of the Schwann cell and had a normal appearance (fig. 7.16). The axon was surrounded by a myelin sheath (fig. 7.16). The myelin sheath was narrow and the outer lamella had not fused to the others (fig. 7.16). Other axons showed myelination at an earlier stage (fig. 7.17) where there were few myelin lamellae and they were less consolidated.

ii) Central zone

The central zone two weeks after treatment showed further fragmentation of the injured myelin and disappearance

of necrotic cell debris (fig. 7.18). Many endoneural tubes were "empty" apart from peripheral myelin fragments and Schwann cell debris (fig. 7.18). The Schwann cell membrane had ruptured but the basal lamina was intact (fig. 7.18).

In other endoneural tubes where the disintegration was less advanced the centre of the endoneural tube was filled with debris (fig. 7.19). The new axons were peripheral and their long axes were parallel to the long axis of the endoneural tube (fig. 7.19).

In regions of new Schwann cells (fig. 7.20) their nuclei were large and active (fig. 7.20). The indication of nuclear activity was the fine granular appearance of the nucleoplasm (fig. 7.20). The Schwann cell was surrounded by newly formed axons (fig. 7.20).

iii) Distal zone

The distal zone showed the same degree of myelin degeneration as the central zone. There was not as much evidence of regeneration as in the central zone (fig. 7.21). Many of the axons showed early stages of Wallerian degeneration (fig. 7.22) in which the axon had been displaced from the normal central position and the myelin sheath was degenerate. The newly formed axons were of small diameter and situated at the periphery of the nerve fibre (fig. 7.22a). The new axons contained many neurofilaments, scattered mitochondria and some membrane bound vesicles (fig. 7.22a).

C Three weeks after treatment

i) Proximal region

Three weeks after treatment the removal of debris was almost completed and frequently the only remnants of the injured nerve fibres were the collapsed, basal laminae (fig. 7.23). In some places the remnants were devoid of content (fig. 7.23) and in others part of the convoluted basal lamina surrounded a small regenerated axons and Schwann cell (fig. 7.23). New basal lamina had been deposited within the old one (fig. 7.24). The fibroblasts were much in evidence (fig. 7.23).

The Schwann cell cytoplasm was also very active and contained many ribosomes, polysomes, rough endoplasmic reticulum cisternae and elongated mitochondria (fig. 7.23). The endoneural tubes were packed with newly formed Schwann cells (fig. 7.25) each of which enclosed one or more new axons (fig. 7.25).

Different stages of myelination of the new axons were seen (fig. 7.24, 7.25). The first stage occurred when the mesaxon or Schwann cell membrane surrounded the axon (fig. 7.25). Several layers of mesaxon were wrapped around another axon (fig. 7.25) but the mesaxon membranes had not fused with one another at this stage to form the characteristic myelin lamellae (fig. 7.25). Around some axons the mesaxon membranes had fused to give the characteristic appearance of myelin (fig. 7.24). The fusion of the lamella was not uniform around the axon (fig. 7.24).

The axon contained microtubules, neurofilaments, a few small vesicles and mitochondria (fig. 7.24).

Fig. 7.24 represents a more advanced stage of regeneration than (fig. 7.25) as the individual Schwann cells within the old endoneural tube showed a complete or incomplete new basal lamina.

The remaining debris was confined to the centre of endoneural tubes (fig. 7.26) and consisted of fragments of degenerate myelin.

ii) Central region

Clearing of necrotic debris was still evident in the central zone three weeks post operatively. There were many macrophages and fibroblasts in the intercellular spaces. The same stages of regeneration seen in the proximal region were seen in the central region of the lesion (fig. 7.27). The new Schwann cells and their axons were partially surrounded by new basal lamina within the old endoneural tube (fig. 7.27). There were collagen fibres between the old and new basal laminae (fig. 7.27). Different stages in myelination were present (fig. 7.27) from early mesaxon elongation to fusion of the lamellae to give mature myelin. The more mature myelin sheaths were narrow (50 nm wide) (fig. 7.27).

The remnants of the injured axons and myelin sheaths were seen in the middle of the endoneural tubes (fig. 7.27, 7.28). The non-myelinated nerve fibres also showed new basal lamina formation (fig. 7.28). There were collagen fibres between the old and new basal laminae of the non-

myelinated nerves (fig. 7.28). The majority of the small non-myelinated axons within the endoneural tube were normal but one axon contained only sparce material and two degenerate organelles (fig. 7.28). Another axon contained only amorphous lipid-like material. The different axons in the non-myelinated endoneural tube showed irregular orientation as the neurofilaments and microtubules were cut in different planes (fig. 7.28).

iii) Distal region

There were less regenerate nerve axons in the distal region than in the central or proximal regions. The regeneration of the nerve fibres was not so advanced in the distal region.

The myelin sheaths forming around the regenerate myelinated nerve axons had not completed the fusion of the myelin lamellae as there were small areas of unfused lamellae in the developing sheath (fig. 7.24). The regenerating axons were at different stages of maturation. The Schwann cells which contained no regenerating axons contained less cytoplasmic organelles (fig. 7.29).

Within the endoneural tube two other Schwann cells were adjacent to the one surrounding the myelin sheath (fig. 7.29). One Schwann cell had extended a cytoplasmic process towards a new axon (fig. 7.29). The old basal lamina had ballooned away from the Schwann cells where new basal lamina formation had occurred (fig. 7.29). The new Schwann cell contained two Golgi bodies and a centriole as well as the usual cell contents (fig. 7.29).

The non-myelinated nerves (fig. 7.30) were also less advanced in their repair processes. Degenerate axons were juxtaposed to new axons (fig. 7.30). The degenerate axons had lost their normal structure and instead were either "empty" or contained irregular membrane-bound vesicles (fig. 7.30).

The Schwann cell cytoplasm contained numerous, membrane-bound vesicles (fig. 7.30) some vesicles were secondary lysosomes and the others were featureless (fig. 7.30).

In places the intercellular spaces were filled with floccular material and a few collagen fibres (fig. 7.30). In other areas the endoneural collagen fibres were better established (fig. 7.29) and the ground substance was not coagulated.

There was more necrotic debris in the distal region than in the central region.

D Four weeks after treatment

The biopsies taken four weeks after treatment showed much regeneration and reconstitution of the nerve fibres. By four weeks the differences between the regions was less distinct. However, in order to retain the same format for description the lesion will be reported in three parts.

i) Proximal region

The proximal region four weeks after treatment was packed with myelinated and non-myelinated nerve fibres (fig. 7.31) which were surrounded by abundant collagen fibres. The limits of the old endoneural tubes could be

seen (fig. 7.31) although the separation of small bundles of new non-myelinated axons away from the myelinated axon was almost complete (fig. 7.31). Only one new myelinated nerve regenerated in each old endoneural tube (fig. 7.31) but several non-myelinated nerve fibres were formed. The non-myelinated nerves contained several axons of small diameter, enclosed by Schwann cell cytoplasm (fig. 7.31, 7.32). The myelinated axon was much larger than the axons in the non-myelinated portions of the old endoneural tube (fig. 7.31). As a consequence of the fragmentation of the old endomysial tubes there were areas packed with small, irregular non-myelinated nerves (fig. 7.32). Each non-myelinated nerve was enclosed by a basal lamina (fig. 7.32). In many non-myelinated fibres the axons were at the periphery of the endoneural tube, juxtaposed to but not enclosed by the Schwann cell (fig. 7.32). In other non-myelinated nerve fibres the Schwann cell cytoplasm had partially or entirely enclosed the axons (fig. 7.31).

Isolated non-myelinated nerve fibres contained up to 50 axons within the endoneural tube (fig. 7.33) all of which were enclosed by Schwann cell cytoplasm.

The correct orientation of all the non-myelinated and myelinated axons had been restored as it was possible to cut true transverse sections.

The non-myelinated and myelinated axons were normal in appearance and contained microtubules, neurofilaments and mitochondria (fig. 7.31, 7.32, 7.33). The myelin

sheaths were also normal with well developed nodes of Ranvier (fig. 7.34) and Schmidt Lanterman incisures.

ii) Central region

The overall picture of the central region was the same as in the proximal region. There were regenerated myelinated and non-myelinated nerves (fig. 7.35, 7.36). The non-myelinated nerve fibres were present in much higher numbers than the myelinated nerve fibres.

As in the proximal region only one regenerated axon in the endoneural tube became myelinated (fig. 7.35) and this axon was much larger than the others. Not all the non-myelinated clusters of axons had separated from the parent endoneural tube (fig. 7.35). The Schwann cells were active and contained Golgi bodies, mitochondria, many ribosomes and rough endoplasmic reticulum (fig. 7.35). Unlike the proximal zone, the central region still contained a little necrotic debris and remnants of degenerate myelin sheaths (fig. 7.36, 7.37). The endoneural collagen fibres were not as dense or as well established as in the proximal zone.

The remnants of the original basal laminae were still present (fig. 7.37).

iii) Distal region

There were more remnants of degenerate nerve fibres in the distal region than in the central region, four weeks post operatively (fig. 7.38, 7.39). Early stages of myelination were present (fig. 7.40)and the myelinated axons (fig. 7.39) were not as large as in the proximal

region (fig. 7.31).

The separation of the new non-myelinated portions from the myelinated nerves in the same endoneural tubes (fig. 7.39) was evident from the deposition of new basal lamina within the old endoneural tube.

Summary of Results

1 One week after treatment

The degenerative changes were still evident and regenerative changes were also seen as the formation of new, fine, axons. It was difficult to distinguish the true non-myelinated axons from the axons in which myelination had not yet commenced. Schwann cells surrounded the necrotic nerve debris and the regenerated axons were confined to the peripheral areas. The basal lamina was still intact in all three regions. Active phagocytes were seen near the nerve fibres. The distal region showed less extensive degenerative changes than the central region. Only a few regenerated axons were seen in the distal region.

2 Two weeks after treatment

Most of the necrotic debris had been removed. The removal of the necrotic debris was associated with an increase in the number of regenerated axons. In many areas a newly formed basal lamina surrounded the Schwann cells. Within the central region some of the endoneural tubes contained no recognisable structures.

3 Three weeks after treatment

The process of myelination had commenced and most of the necrotic debris had been removed from all three regions. These changes were associated with an increase in number of regenerated axons. In the distal region empty endoneural tubes were seen.

4 Four weeks after treatment

The reconstitution of the nerve fasciculus was associated with an increase in the number of the regenerated axons within the three regions and the differences between the three regions had decreased. Schwann cells containing only one large axon showed an advanced stage of myelination and was surrounded by a new basal lamina.

In all three regions the old basal lamina showed different stages of disintegration which was associated with separation of new Schwann cells and their axons. A few remnants of necrotic debris were still present in some endoneural tubes of the central and distal regions.

Discussion

Although the degeneration of the sciatic nerve was rapid and extensive, regeneration had started by one week after treatment. Mira (1979) also showed that the regeneration of peripheral nerve fibres damaged by freezing was faster than that achieved by division or crush.

The regenerated axons occupied the peripheral region of the endoneural tubes between the Schwann cells and the intact basal lamina. This was thought to be due to

contact guidance and contact inhibition. As early as 1912, Ranson had seen, in the light microscope, that regenerating axons followed the course set by columns of Schwann cells. This has also been reported in the electron microscope (Nathanial and Pease, 1963b).

Contact inhibition was thought to influence peripheral axon regeneration by preventing the new axon from penetrating the necrotic debris. In this study the large, regenerated axons were not found close to the debris. In tissue culture the presence of cells other than Schwann cells in the endoneural tube has been shown to inhibit the centrifugal axonal growth (Dunn, 1971).

By one week the necrotic debris within the endoneural tube had shrunk as in typical Wallerian degeneration (Morris et al., 1972 a and b). The appearance of the debris was thought to be a result of local enzyme action (Hallpike, 1976). Associated with the removal of debris were the old Schwann cells which contained membrane bound vesicles and active Golgi apparatus. Myelin fragments were also seen in membrane bound vesicles within the old Schwann cells. It is thought that the old Schwann cells were actively participating in the first stage in the removal of necrotic debris from the endoneural tube. Gibson (1979) also concluded that Schwann cells were involved in degradation and digestion of necrotic nerve debris after crush injury. At no time were any of the usual phagocytes seen within the endoneural tubes.

Macrophages found between the endoneural tubes contained

membrane bound vesicles with electron dense contents. The membrane bound vesicles within the macrophages were of the same appearance as free, floating vesicles surrounding the endoneural tubes. It is thought that the Schwann cells within the endoneural tubes had digested and degraded the necrotic debris and expelled them through the basal lamina to be picked up by the macrophages. This hypothesis is supported by the work of O'Dally and Imaeda (1967) on degenerate cutaneous nerves.

The second type of Schwann cell within the endoneural tubes were newly formed and are thought to have originated in undamaged areas. These new Schwann cells, which had migrated into the damaged area, will be responsible for myelinization of the newly formed axons. Gutters formed in the Schwann cells in which the axons became embedded. A mesaxon was formed as the first stage of remyelination.

In large diameter endoneural tubes the number of Schwann cells was greater than in the smaller diameter tubes. The reasons for this may be purely physical in that there was more space within the larger tubes or the numbers of Schwann cells may be related to a stimulous from the debris. The greater the amount of debris the more Schwann cells will be needed for phagocytosis and vice versa.

In the distal zone one week after treatment the process of Wallerian degeneration was still slow. Related to the slow rate of degeneration was the slow

Schwann cell activity compared to the central and proximal regions. This also indicated an intimate association between accumulation of endoneural debris and Schwann cell proliferation. It is not possible at this stage to say whether the stimulation for Schwann cell proliferation is due to the absence of an intact axon or the presence of axonal debris.

In some endoneural tubes the Schwann cells were degenerate. There are several possible reasons for the break down of Schwann cells. First, the Schwann cells may just be at the end of their lives after an active role in phagocytosis. It has been shown in tissue culture that after nerve cells have been treated with x-rays, the Schwann cells degenerate in response to massive myelin breakdown. Alternatively, if the axons fail to regenerate or undergo secondary degeneration, the axon-Schwann cell relationship could not be established. Liu (1974) showed that the presence of axons was essential for the existance of Schwann cells. The intimate association between Schwann cell and axon is still little understood. The degeneration of Schwann cells led to the appearance of empty endoneural tubes.

By two weeks in the central zone most of the endoneural tubes showed regenerated axons and in some myelination was well advanced. The progress of myelination of regenerated axons proceeded in the same way as in developing myelinated nerves (Bloom and Fawcelt, 1968). The progress of myelination was related to axon size as the larger

axons were at a more advanced stage of myelination than the smaller axons. Mathews (1968) showed that myelination could not be initiated until the axon had attained a certain diameter.

As the regeneration proceeded the Schwann cells enclosed either a single large axon undergoing myelination or several fine unmyelinated axons. Within an old endoneural tube would be more than one Schwann cell enclosing regenerated axons. Each Schwann cell with its new axons was progressively separated from its neighbours, within the old endomysial tube, by a basal lamina. Several workers have attributed an important role to the persistent basement membrane in maintaining the structural integrity during degeneration (Thomas, 1964a; Nathaniel and Pearse, 1963b). The persistent, original basal lamina provides a tube which encloses proliferating Schwann cells and also provides pathways which guide regenerating axons to their terminations. The formation of new basal lamina within the confines of the original basement membrane has also been described by Nathaniel and Pearse (1963b) and Thomas (1964b). The origin of the new basal lamina is thought to be the newly formed Schwann cells, thus ensuring that each Schwann cell and its axons is enclosed by a basal lamina. The new basal lamina surrounding each Schwann cell would increase the surface area for the passage of nutrients and other substances across the selective permeable membrane.

Associated with the formation of a new basal lamina was the appearance of collagen fibres between the old and new basal lamina. This appearance has led observers to propose that the collagen fibres were produced by the Schwann cells (Gamble and Rosemary, 1964; Thomas, 1976; Mustafa, 1978). From the present observations there was no evidence of collagen fibre production by the Schwann cells. It is thought that these collagen bundles originate from endoneural fibroblasts at the time of the appearance of collagen fibres close to the new basal lamina, there was an increase in active fibroblasts around the regenerated nerve units. The collagen fibres are thought to have passed through the degenerating old basal lamina in order to establish their intimate association with the new basal lamina. If the Schwann cells were active in collagen fibre production, the collagen fibre sub-units would have to pass through the basal lamina, which is most unlikely. It would also make fibroblasts in nerve tissue redundant. Thomas (1966) was able to demonstrate that cords of Schwann cells were enclosed by connective tissue outgrowth from the perineurium and endoneurium, containing fibroblasts, blood vessels, collagen fibres, macrophages and polymorphs.

Unmyelinated fibres appear to have the capacity to regenerate more rapidly than myelinated fibres (Bray, Peyronnard and Aguayo, 1972; Dyck and Hopkins, 1972; Aguayo, Peyronnard and Bray, 1973). For example, regeneration was evident in the rabbit anterior mesenteric

nerve as early as two days after transection. Initially up to 200 axons were associated with one Schwann cell, but this number declined as axon groups were divided up to progressively smaller numbers by Schwann cells (Bray and Aguayo, 1974). Bray and Aguayo (1974) demonstrated a gradual loss of redundant axonal sprouts.

It was difficult to decide, in the present study, whether the non-myelinated nerves were true non-myelinated nerves or potentially myelinated nerves which had not yet commenced myelination. King and Thomas (1971) examined the axon-Schwann cell relationship in the regenerated recurrent laryngeal nerve. They also found that the unmyelinated axons were usually immediately juxtaposed to a myelinated fibre being arranged circumferentially or crescentically around the central myelinated fibre. This configuration suggests that these "unmyelinated" nerve fibres have been separated by new basal lamina formation from a central Schwann cell, all of which were contained within the old endoneural tube. Whether or not these "unmyelinated fibres" ever become myelinated with time requires further investigation. Thomas and King (1971) said that they considered the Schwann cells associated with the unmyelinated axons to be derived from Schwann cells previously associated with a myelinated axon. The author does not think it is right to call these fibres true unmyelinated nerves without further study to investigate whether or not they do become myelinated with time and restoration of function.

It should be remembered that the unmyelinated nerve fibres were more resistant to freezing than the myelinated fibres. This fact could be used to support both points of view. First that as the non-myelinated fibres were less damaged there would be less need for extensive regeneration of unmyelinated fibres so the ones observed are slowly developing unmyelinated nerves. Alternatively, as the myelinated fibres are more susceptible to freezing, less myelin is laid down as a protective measure. It is an interesting problem which requires much further investigation.

CHAPTER 8

LIGHT MICROSCOPE OBSERVATIONS OF THE EFFECT OF CRYOSURGERY ON HORSE SKIN.

Introduction

It is a well known clinical problem that a relatively mild insult to the skin of the horse produces extensive connective tissue proliferation. Although granulation tissue is indispensible in the repair of most wounds in horses, the development of exuberant granulation tissue is a frequent and universal complication of wound healing. The exuberant granulation tissue has the same characteristics as neoplastic tissue (Boyd and Britton, 1972) and the basic cell is embryonic. The exuberant granulation tissue overgrows the wound in rough grape-like masses known as granulomas which resemble benign neoplasms. The subsequent uncontrolled tumour-like growth is very susceptible to infection and mechanical trauma.

Exuberant granulation tissue is a frequent complication of conventional surgical operations in the horse (Silver, 1979). However, previous studies on the effect of cryotherapy on different skin lesions in the horse have shown ordered healing with no such complications (Joyce, 1975; Farris, 1976; Twidale, 1977).

In order to remedy the scarcity of detailed light and electron microscopic studies on the effect of cryosurgery on horse skin, the following study was initiated. The following chapters are a report of a light and electron microscopic investigation of normal horse skin and the

healing process after cryosurgery.

Materials and Methods

A black horse, 5 years old and of 430 kg body weight was used in the study. After premedication with acetylpromazine general anaesthesia was induced by an intravenous injection of a solution of thiopentone sodium B.P. Narcosis was maintained by the inhalation of a halothane-nitrous oxide-oxygen mixture from a Boyles anaesthetic apparatus and circle absorber, via a cuffed endotracheal tube.

Pieces of normal skin 5 cm in diameter were excised from one side of the horse. The biopsies were taken from the following three sites; i) the lower lateral abdomen, ii) lateral chest wall and iii) the lower medial aspect of the hind limb. Standard aseptic surgical procedures were employed. On the contralateral side of the horse areas of skin of the same dimensions and equal sites were frozen to below $-20^{o}C$ using a CE8 Cryosurgical Unit and allowed to thaw. Freezing was monitored by thermocouples and a Downes Tissue Temperature Recorder.

Slice biopsy specimens were taken, under general anaesthesia, from normal skin of the cryosurgically induced wounds at intervals of 20 minutes, 24 hours, 7, 14, 21 and 28 days.

The normal skin biopsies were retained as controls and prepared for examination in the same manner as the treated biopsy samples. Each biopsy was divided into two parts and one was prepared for electron microscopy as in

chapter 3. The second part was fixed in Bouin fixative for two days and prepared for light microscopy (chapter 2). The stains used for light microscopy were i) H and E in order to show general cellular features; ii) Mason's trichrome for connective tissue fibres; iii) Martius yellow, scarlet soluble blue stain (M.S.B.) as a selective stain for fibrin; iv) Mason-Fontana stain for melanin.

Observations

A Normal skin

The biopsies of normal skin from the three different sites showed the same histological features. The horse skin consisted of a surface epithelium or epidermis and an underlying connective tissue layer, the dermis (fig. 8.1). The dermis and epidermis were separated by the narrow basement membrane (fig. 8.1). The epidermis was a keratinised, stratified squamous epithelium (fig. 8.1) Rete pegs were not obvious. The majority of the epidermal cells contained melanin pigments which decreased in number towards the surface (fig. 8.1). The epidermis could be divided into four layers; a stratum lucidum was not observed (fig. 8.1).

The basal cell layer or stratum germitivum consisted of a single row of columnar cells with their long axes perpendicular to the basement membrane (fig. 8.2). The basal cells contained oval, basophilic nuclei with round conspicuous nucleoli (fig. 8.3). The cytoplasm was eosinophilic and contained many rounded melanin granules (fig. 8.1, 8.3).

The stratum spinosum varied from three to ten cell layers thick and the cells were large and polyhedral in outline (fig. 8.1, 8.2). The nuclei were basophilic and the cytoplasm eosinophilic. There appeared to be fewer melanin granules in the cytoplasm but this probably indicated a less concentrated arrangement (fig. 8.1).

The stratum granulosum was two to four cell layers thick and the cells were flattened to a fusiform shape (fig. 8.1). The cells of the stratum granulosum contained many keratohyalin granules.

The outermost layer was the stratum corneum and consisted of several layers of flattened squames (fig. 8.2). The cells contained no organelles.

The dermis of the horse was thick and could be divided into two layers (fig. 8.4). The superficial layer, situated directly underneath the epidermis consisted of loose connective tissue bundles interspersed with collagen fibres (fig. 8.1, 8.4). There were several thin, spindle shaped fibroblasts, mast cells and a few fine rounded blood capillaries in the superficial layer (fig. 8.1). In many areas fine bundles of rectipapillae muscle fibres were also observed (fig. 8.4). The connective tissue continued towards the deeper layer where there were more collagen bundles (fig. 8.5). The collagen bundles were thicker and there were fewer fibroblasts (fig. 8.5). Between the collagen bundles were several medium sized arteries and veins (fig. 8.5). Also in the deeper connective tissue layer were several skin appendages such

as hair follicles, sebaceous and sweat glands (fig. 8.4). The sweat glands were seen in the middle and deep levels of the dermis, and were of the apocrine type (fig. 8.4, 8.6). These glands consisted of numerous spherical masses of tubules lined with cuboidal epithelial cells, which contained round peripheral basophilic nuclei (fig. 8.6). The nuclei were surrounded by abundant eosinophilic cytoplasm containing fine granules. These glands were connected to the surface by narrow excretory ducts.

The sebaceous glands were situated at the upper part of the deep dermis near the roots of the hair follicles (fig. 8.4, 8.7). These glands varied in size and were comprised of up to three lobules, which were connected to the neck of the hair follicle by a short excretory duct (fig. 8.8). The body of the glands consisted of large cuboidal secretory cells, whose cytoplasm contained many fine rounded vacuoles (fig. 8.7, 8.8). The nuclei were large, basophilic and rounded and occupied the central part of the cell (fig. 8.7).

The hair of the horse was distinguished by the rectangular medullary cells separated by prominent intracellular substances (fig. 8.9). The histological structure of the hair follicles was of typical mammalian type and consisted of a hair papilla and a hair root (fig. 8.9, 8.10). The hair papilla consisted of several layers of epithelial cells, concentrically arranged. The majority of the cells contained melanin granules. The root of the hair follicle was cylindrical and also

consisted of several layers of cells (fig. 8.9). The central part, or medulla, consisted of several layers of rectangular cells (fig. 8.9). The appearance of the medullary cells was dependent on the level within the follicle. Towards the surface, the medulla disappeared, leaving keratinised cells (fig. 8.11).

The medulla was surrounded by a cortical layer which was acidophilic and contained tricohyalin (fig. 8.12). The inner root sheath was thin and consisted of a layer of one to two elongated cells thick, and in some hair follicles this layer was not fully recognised (fig. 8.12). Huxley's layer showed one to two rounded cells containing a prominant basophilic rounded nucleus. Henly's layer was of one to two cells thick. The external root sheath was two to four cells thick, depending on the size of the hair follicle. The hair follicle was surrounded by a thin acidophilic layer, the glassy layer (fig. 8.12), external to which was a thin membrane of connective tissue fibres.

B Twenty minutes after treatment

On gross examination twenty minutes after treatment, the treated area was swollen and well circumscribed and was covered by a thin film of exudate. The lesion showed that the central zone directly under the cryoprobe had suffered greater injury than the area at the boundaries of the lesion. In the central area, the epidermis showed localised epithelial slough which resulted in disruption of epidermal continuity. The damage extended to the dermis.

In the light microscope, the most conspicuous feature of the central region of the biopsy was the disruption of epidermal continuity (fig. 8.13). The hair follicles were also disrupted (fig. 8.13). The connective tissue region immediately below the epidermis contained oedematous fluid interspersed between the collagen bundles (fig. 8.13).

The blood vessels of the dermis immediately under the point of the cryoprobe application were severely congested (fig. 8.14). The fibroblasts were degenerate with pyknotic nuclei (fig. 8.14). The sweat glands showed much more severe injury than the sebaceous glands (fig. 8.15). The sebaceous gland cells were still intact 20 minutes post operatively (fig. 8.16) but the nuclei were irregular and pyknotic. The sweat gland tubules had collapsed (fig. 8.15) and the cells had ruptured (fig. 8.17). The sweat glands consisted of a mass of cell debris containing irregular, pyknotic nuclei surrounded by the intact connective tissue bundles (fig. 8.17).

The peripheral region showed less injury but there was localised disruption of the epidermis (fig. 8.18). Immediately beneath the disrupted epidermis the connective tissue demonstrated extensive disruption of the collagen bundle architecture (fig. 8.18). The disturbed collagen bundles were surrounded by oedematous fluid (fig. 8.18, 8.19).

The surface of the intact epidermis was covered by oedematous exudate containing many red blood cells (fig. 8.19). The stratum corneum underlying the exudate

showed separation and disruption (fig. 8.19).

The sebaceous glands in the peripheral region were only slightly affected at 20 minutes post treatment but the sweat glands were damaged (fig. 8.20). The sweat glands were much more susceptible to cold injury than the sebaceous glands (fig. 8.20). At a considerable distance (approximately 1 cm) from the centre of the lesion, where other structures in the dermis had a normal appearance, the sweat glands were injured.

The hair follicles showed varying degrees of injury from total destruction in the central region (fig. 8.13) to just mild infiltration of oedematous fluid into the root of the papilla in the peripheral region (fig. 8.21).

C Twenty four hours after treatment

The gross examination of the lesion twenty four hours after treatment showed swelling and a covering of thick, moist, brownish exudate.

In the light microscope, the biopsy showed complete disruption of the epidermis (fig. 8.22) which had been replaced by a mass of tissue exudate and debris. The moist scab contained fragments of epidermis (fig. 8.22). Adjacent to the scab the epidermis was severely injured but still recognisable (fig. 8.22).

There was a vast infiltration of polymorphs into the region immediately underlying the scab and adjacent injured epidermis (fig. 8.22). Beneath this there was a dense band of connective tissue at a slightly less advanced stage of degeneration (fig. 8.22). This layer contained

scattered polymorphs (fig. 8.27) which were more concentrated in the peripheral region (fig. 8.22). The more central region of the layer had a hyalinised appearance (fig. 8.22). The blood vessels were damaged in this intermediate layer. The innermost layer of the dermis (fig. 8.22) showed separation of the collagen bundles (fig. 8.23). The layers of dermis showing different degrees of injury were well defined (fig. 8.22).

Towards the periphery of the lesion areas of fibrin were seen (fig. 8.24, 8.26). The fibrin filled masses were interspersed between clumps of cells and occasional collagen bundles (fig. 8.24). It was difficult to distinguish between the different cell types in this area (fig. 8.24).

In the centre of the lesion there was total destruction of the hair follicles, sweat glands, sebaceous glands, blood vessels and collagen bundles (fig. 8.22). Peripheral to the central zone the various skin components were recognisable but showed varying degrees of injury (fig. 8.22). The blood vessels contained microthrombi (fig. 8.25); the hair follicles showed varying degrees of injury (fig. 8.22, 8.27, 8.29) and the sweat gland (fig. 8.28) tubules were collapsed and the cells ruptured.

The centre of the lesion was shown in fig. 8.22 and peripheral to this region the skin showed local blisters approximately 0·5 - 2 mm in diameter (fig. 8.30). The epidermis, which was still intact had separated from the dermis (fig. 8.30). Between the elevated epidermis and

the dermis was an area of acidophilic coagulated, oedematous fluid (fig. 8.30). There was a dense band of polymorphs which had infiltrated the connective tissue immediately beneath the coagulated mass which made up the centre of the blister. The hair follicles and sebaceous glands were slightly damaged in this region (fig. 8.30) although the sweat glands did show some degeneration.

Further away from the centre of the lesion early stages of blister formation was seen. These occurred in localised regions and the first evidence of blister formation was the separation of the epidermis and the dermis (fig. 8.31). In this micrograph (fig. 8.31) the area immediately adjacent to a hair follicle showed the greater degree of separation. The area created by the separation contained oedematous fluid and many red blood cells (fig. 8.31). The epidermis was still intact (fig. 8.32). Polymorphs had accumulated at the junction of the oedematous fluid (fig. 8.32) and the dermis. The cell inclusions of the polymorphs were conspicuous (fig. 8.32).

At the extreme periphery of the biopsy the skin had a normal appearance (fig. 8.33). The only evidence of injury in this region was seen in the blood vessels which were congested (fig. 8.34) and the sweat glands. The sweat glands showed disorganisation of the normal internal structure (fig. 8.34) and some of the cells had ruptured.

D One week after treatment

By the end of the first week the wound had contracted leaving a well defined dry necrotic mass which was dark brown. The contraction had resulted in a scab half the size of the original injury. The scab was surrounded by a thin rim of smooth purple skin which was hairless.

Sections of the biopsy revealed a central area of amorphous, hyalin-like material (fig. 8.32) which contained no recognisable components. The edges of the less damaged peripheral region had started to grow towards the centre of the lesion underneath the scab and raising it above the level of the skin (fig. 8.35). The contraction of the wound will also have contributed to raising the scab.

Beneath the dense scab was a layer of loosely packed necrotic debris (fig. 8.35).

In the area peripheral to the scab some of the hair follicles still showed degenerative changes, whilst others had indications of regeneration (fig. 8.35, 8.36). The regeneration of the hair follicles was seen as a dense arrangement of basophillic cells (fig. 8.36). This was in contrast to a neighbouring hair follicle (fig. 8.36) where the cells were loosely packed and very pale staining (fig. 8.36).

The epithelium of the peripheral region beneath the scab showed a proliferation of cells which extended well into the dermis (fig. 8.35). The downgrowth of epidermal cells was flanked by less extensive cellular growth (fig. 8.37). Immediately below the scab the

collagen bundles were totally destroyed,at the immediate periphery there were injured collagen bundles which were amorphous and discrete. Interspersed between the collagen bundles were fibroblasts, macrophages and other cell types (fig. 8.38). The macrophages were frequently observed close to the bundles of regenerated collagen (fig. 8.38). Newly formed fine collagen fibres were also present in this area (fig. 8.38).

Some of the blood vessels in the region adjacent to the scab showed signs of organisation (fig. 8.39) the tip of the blood vessel was partially occluded by an organised thrombus (fig. 8.39). Fibroblasts had invaded the thrombus (fig. 8.39) and thin strands of collagen had been deposited. Large numbers of polymorphs had migrated to the area juxtaposed to the thrombus within the injured blood vessel (fig. 8.39).

At the extremity of the scab the epidermis had more than doubled in thickness (fig. 8.40). The thickened epidermis showed rete peg formation which was not seen in normal horse skin (fig. 8.41). Within the thickened epidermis were many cells showing clear perinuclear regions (fig. 8.41). The epidermal cells in the hypertrophic epidermis contained no melanin pigment (fig. 8.41). The basal cell layer was well defined but the other epidermal cells were not organised into the normal strata (fig. 8.41). The dermis lying beneath this region contained many fibroblasts, collagen bundles (fig. 8.41) and regenerating hair follicles (fig. 8.40).

The regenerating hair follicles showed active germinal cells with prominent basophilic nuclei (fig. 8.41). Only a few hair follicles were associated with sebaceous glands (fig. 8.40). The sebaceous glands were small (fig. 8.40). No sweat glands were seen in this region (fig. 8.40).

The collagen bundles of the deep dermis in the region of the thickened epidermis were organised into undulating bands (fig. 8.42). These bands of densely packed collagen fibres were separated by less organised collagen fibres and fibroblasts (fig. 8.42).

The peripheral skin was normal.

E Two weeks after treatment

By the second week the lesion had further contracted and the scab had been sloughed off. A few dry scab crusts were still attached to the central region of the wound. The area of regenerated tissue had increased and it divided the central region from the intact peripheral areas. The regenerated area was pink and hair-less. The hair shafts of the area adjacent to the regenerated region had grey bases.

The centre of the lesion consisted of the scab which was loosely attached to the underlying connective tissue (fig. 8.43). Some regions of the scab contained densely packed degenerate collagen bundles (fig. 8.44). The underlying area was packed with polymorphs, fibroblasts, macrophages and other undifferentiated cells (fig. 8.45) forming granulation tissue. The cells were surrounded

by unorganised collagen fibres and bundles (fig. 8.45). In other areas underlying the scab the collagen bundles were thicker and more organised (fig. 8.44, 8.46).

The edge of the scab was still firmly attached to the hypertrophic epidermis (fig. 8.47). The epidermis had grown under the scab towards the centre of the lesion (fig. 8.47). In this region the epidermis was much thicker than normal and showed well developed rete pegs (fig. 8.47) only the epidermis at the region of attachment to the edge of the scab contained non-epithelial cells (fig. 8.47). These cells consisted of a central nucleus surrounded by clear cytoplasm (fig. 8.47).

The basal cell layer of the thickened region of epidermis was well defined (fig. 8.48). Both the basal cell layer and the stratum spinosum cells contained conspicuous nuclei with prominent nucleoli (fig. 8.48). The most noteworthy feature of the epidermis was the complete lack of melanin granules in any of the regenerating epidermal cells (fig. 8.48).

In the hair-less zone the normal division of the dermis into two distinct regions had been lost (fig. 8.49). The thick collagen bundles which were seen in the deep dermis in normal skin, were found close to the rete pegs (fig. 8.49).

The injured blood vessels had been sealed off by a completely organised thrombus (fig. 8.50) leaving concentric layers of fibroblasts and collagen. Newly formed fine blood vessels were established in close proximity to the

occluded vessel (fig. 8.50). The new blood vessels were only a quarter of the diameter of the old vessel (fig. 8.50).

The most significant feature of the outermost region of the biopsy was the regeneration of the hair follicles (fig. 8.51). The regenerated hair follicles were normal in most aspects apart from a complete lack of melanin granules (fig. 8.51). The cell layers of the hair follicles were well defined and active (fig. 8.51). The sebaceous glands were normal (fig. 8.51).

There was a noticeable increase in the bundles of collagen fibres and small blood vessels around the unpigmented hair follicles (fig. 8.51).

In another region of the periphery of the lesion, the hair follicles were at a less advanced stage of regeneration (fig. 8.52). The cell layers of the follicles were less organised (fig. 8.52). At the periphery of some of the hair follicles were small groups of sebaceous gland cells (fig. 8.52).

There were no sweat glands in any of the areas of the biopsy examined two weeks after treatment.

F Three weeks after treatment

By three weeks post operatively, the healing process was well advanced and the scab had been completely sloughed off. The lesion was round and had reconstituted well with no elevation of the skin. The regenerated skin was pale pink and contained a few short, white hairs at the boundary of the regenerated epidermis and the unaffected

skin; the hairs were a mixture of normal brown hairs, white hairs and hairs that were brown at the tip and white at the base.

The surface of the most central part of the lesion consisted of necrotic connective tissue debris (fig. 8.53) which covered the regenerating epidermis and undamaged connective tissue. The epidermis had proliferated towards the centre of the lesion but the covering was not complete. In the centre of the lesion where there was no epidermal covering, the granulation tissue contained round discrete areas of degenerate collagen bundles (fig. 8.54).

At the point of proliferation of the epidermis across the surface of the wound many mitotic figures were seen within the epidermis (fig. 8.55). The epidermis was hypertrophic and contained localised accumulations of non-epithelial cells (fig. 8.53). Towards the outer edge of the central zone the regenerated epidermis showed differentiation into the few normal strata (fig. 8.56). There was a complete absence of melanin within the epidermal cells (fig. 8.54, 8.56).

The granulation tissue in the centre of the lesion contained large numbers of small blood vessels (fig. 8.53). The majority of the blood vessels were orientated perpendicular to the surface (fig. 8.53). Immediately beneath the necrotic debris at the centre of the lesion the granulation tissue consisted mainly of fibroblasts, polymorphs and occasional macrophages (fig. 8.57) interspersed with collagen fibre bundles. The deeper

connective tissue contained mainly densely packed bundles of collagen and fibroblasts (fig. 8.58).

There were no skin appendages such as hair follicles or sweat glands in the centremost part of the lesion.

Towards the periphery the hair follicles were regenerated and the cells of the root were active (fig. 8.59). The activity of the cells was indicated by large nuclei containing prominent nucleoli (fig. 8.59). The absence of melanin was conspicuous (fig. 8.59). The structures associated with hair follicles, such as the sebaceous glands and smooth muscle had regenerated.

It was not until the extreme periphery of the lesion was examined that sweat glands were observed (fig. 8.60).

Also, towards the periphery of the lesion discrete groups of cells were seen within the connective tissue (fig. 8.61). The cells were either triangular or polygonal in outline with a centrally placed nuclei. Most of these cells were filled with homogeneous cytoplasm (fig. 8.61). There was a connective tissue capsule surrounding the cells (fig. 8.61).

The epidermis in the peripheral region was normal apart from the complete lack of melanin granules.

G Four weeks after treatment

The lesion had healed by the fourth week and the wound was well constituted. The size of the wound was only a quarter of the treated area. The central part was pink, smooth and hairless. Surrounding this area was a zone

containing a few white hairs.

The centre of the biopsy consisted of a regenerated epithelial layer covering a layer of connective tissue (fig. 8.62). The epidermis was thick with broad rete pegs (fig. 8.62). The normal stratification was seen with a gradual transition from stratum spinosum to stratum granulosum (fig. 8.62). There was a conspicuous lack of melanin granules. Within the epidermis was a circular keratin pearl (fig. 8.62). The keratin pearls were less frequent towards the periphery of the biopsy. The stratum corneum was relatively thin. There were many non-epithelial cells within the epidermis (fig. 8.62).

The connective tissue immediately beneath the epidermis (fig. 8.62) contained numerous fibroblasts, small blood capillaries, a few lymphocytes and collagen fibres. The deeper dermis (fig. 8.63) was largely composed of dense bundles of collagen fibres which were randomly orientated. There were no hair follicles, sweat glands or sebaceous glands in this region.

The epidermis was not as thick in the more peripheral region (fig. 8.64) and regenerated hair follicles were present (fig.8.64). The hair follicle cells contained no melanin pigment (fig. 8.64). The associated sebaceous glands were well developed and normal (fig. 8.64). There were no sweat glands in this region.

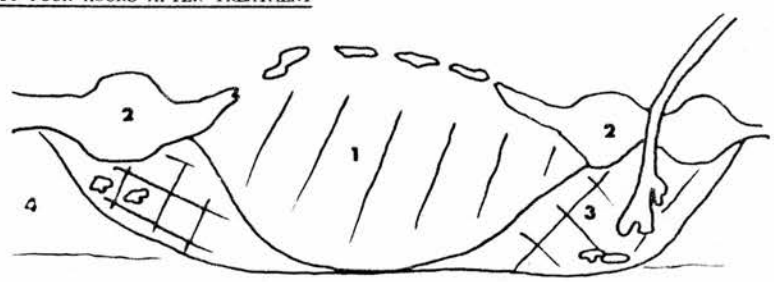
At the extremity of the biopsy sweat glands were present as well as hair follicles and sebaceous glands. The epidermis was normal.

SUMMARY OF RESULTS

TWENTY MINUTES AFTER TREATMENT

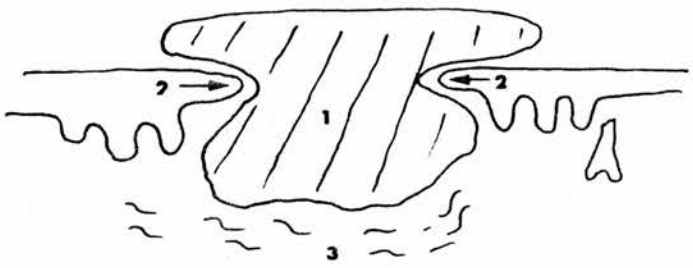
1. CENTRAL ZONE DAMAGED
2. PERIPHERAL REGION SHOWED LESS INJURY
3. SWEAT GLANDS IN PERIPHERAL REGION INJURED.

TWENTY FOUR HOURS AFTER TREATMENT



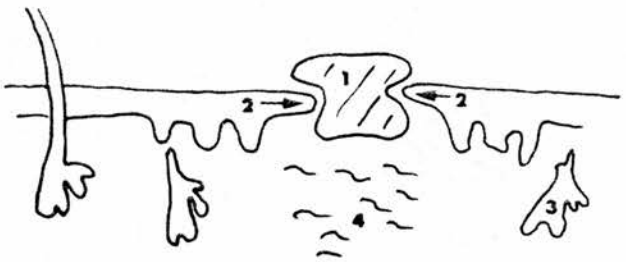
1. CENTRAL NECROTIC ZONE
2. BLISTERS IN TRANSITIONAL ZONE
3. TRANSITIONAL ZONE CONNECTIVE TISSUE
4. PERIPHERAL ARCH LESS AFFECTED

ONE WEEK AFTER TREATMENT



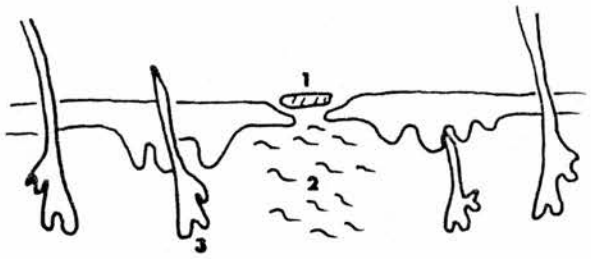
1. NECROTIC AREA
2. EPIDERMIS PROLIFERATING UNDER SCAB TOWARDS CENTRE
3. THICK COLLAGEN BUNDLES UNDER SCAB

TWO WEEKS AFTER TREATMENT



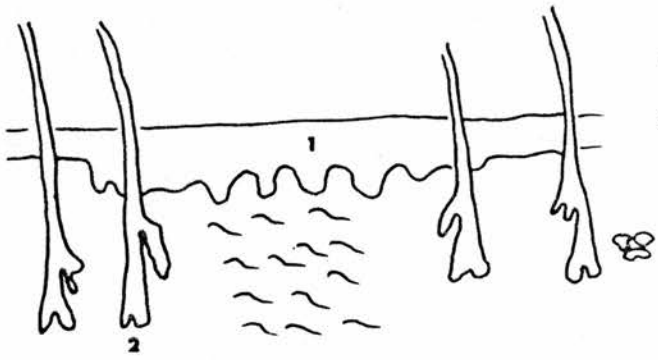
1. SMALL NECROTIC SCAB
2. REGENERATING EPIDERMIS
3. REGENERATING HAIR FOLLICLES
4. THICK SCAR TISSUE

THREE WEEKS AFTER TREATMENT



1. FINE SCAB
2. SCAR TISSUE
3. REGENERATING HAIR FOLLICLE

FOUR WEEKS AFTER TREATMENT



1. SCAR AREA DEVOID OF HAIRS OR SWEAT GLANDS
2. REGENERATED HAIRS ARE WHITE

Discussion

The present study showed uncomplicated healing and a good cosmetic recovery of horse skin after cryosurgery. Clinical studies have also shown a good response after cryosurgery of different skin lesions (Joyce, 1975; Farris, 1976; Twidale, 1977).

The early changes after treatment were similar to those of other tissues injured by extreme cold. The different cell types in the horse skin showed pyknotic nuclei, irregular granular cytoplasm and variable degrees of distortion. The blood vessels were damaged with microthrombi formation. One result of injured blood vessels was thought to be the profuse production of oedematous fluid (Kulka, 1965).

The localised sloughing of the epidermis after treatment was thought to be due to the contact of the cryoprobe with the epidermis. The physical bond would cause rupture of the thin epidermis. The profuse oedematous fluid might also have a mechanical influence. The necrosis of the epidermis, dermis and skin appendages was consistent with the findings of Zacarian and Adham (1966) with frozen dog skin. However, Zacarian et al (1966) did not observe such total necrosis until three days after freezing with a liquid nitrogen cooled copper disc. In the present study the same degree of necrosis was seen within 24 hours. It is thought that the continuous liquid nitrogen device was more efficient at producing extreme tissue damage.

The necrotic zone was well circumscribed by a band of phagocytes and polymorphs. The injury to the epidermis was more extensive than in the underlying structures. This could be a result of the protective properties of the epidermis (Zacarian, 1969) or the fact that dermal cells were less sensitive to cold injury than the epidermal cells. The presence of numerous collagen bundles which have shown little reaction to cold injury in the earlier studies might provide a greater protective factor than the epidermal cells.

The most significant observation in the present study was the extensive proliferation of a thick regenerating epidermis across the surface of the damaged area. Silver (1973) showed a thin layer, two cells thick, migrating across a damaged area after excision of a region of whole skin. The increase in thickness did not start until the thin epidermal covering was complete (Silver, 1973). The thin layer of epidermis was very susceptible to secondary injury (Silver, 1973), which increased the likelihood of poor healing. It seems probable that a thin layer of epidermis would be less likely to control exuberant connective tissue proliferation. In the cryolesion the proliferation and thickness of the regenerating epidermis was established before proliferating across the wound surface. Indeed, the regenerated epidermis was considerably thicker than the normal epidermis.

The contraction of excised wounds from different regions of the horse such as flank and limb, was not uniform

(Walton and Neil, 1972). Clinical studies (Aldeen and Borthwick, 1980) showed uniform and rapid contraction after cryosurgically induced wounds of the chest, abdomen and limb. It is well known that the speed and mechanism of wound healing is intimately related to wound contraction. Dehydration has been suggested as a possible factor (Cuthbertson, 1959) and in the present study there was fast hyalinisation and drying of the necrotic region. The dehydration could also cause the surrounding connective tissue fibres to fill the gap surrounding the decreasing necrotic mass.

Other workers (Abercrombie, James and Newcomb, 1960; Walter and Isreal, 1974) have suggested that newly formed granulation tissue was responsible for wound contraction. However, wounds have been shown to contract without new connective tissue formation in scorbutic conditions (Wilhelm, 1977). Watts, Grillo and Gross (1958) showed that removal of newly formed connective tissue did not affect wound contraction. Fibroblasts and also modified fibroblasts, have been shown to aid wound contraction in rat skin (Gabbiani *et al.*, 1972) and in man (Ryan *et al.*, 1977).

It is most probable that wound contraction results from a combination of several factors. In the present study wound contraction was good and uniform. A failure of wound contraction in the horse may well be an additional factor in the proliferation of exuberant granulation tissue. The gap normally filled by wound contraction

would be filled with granulation tissue which continues to proliferate once the wound is filled. The resistance of collagen fibres to a cryosurgical injury may also be an important factor in the successful contraction seen in the present study.

The blisters that formed in the peripheral epidermis were similar histologically to those seen in man and the dog (Pearson, 1964; Zacarian et al., 1966). However, in the present study their development was slower than observed by Pearson, 1964; and Zacarian et al. (1966).

The sweat glands were particularly sensitive to the cold treatment. In the peripheral regions where there was little injury to the surrounding structures, the sweat glands were disrupted. This is thought to be due to several factors. The sweat gland cells contain a high proportion of water (Luyet and Hartung, 1944; Simpson, 1971) and consequent injury by freezing and thawing is very likely. The sweat glands of horses contain very high concentrations of sodium, potassium, calcium, magnesium and chloride ions (Carlson and Ocen, 1979). During the early stages of freezing and ice crystal formation, the concentration of these ions might have increased to a fatal level.

Extensive ice crystal formation within the secretory granules could have caused rupture of cell membranes. Ice crystal formation within the excretory ducts may also have caused mechanical injury. The sebaceous glands, the cells of which contain less water, were less damaged.

There were a few intact hair follicles at the periphery of the wound associated with regenerated epidermis. These hair follicles were thought to have originated from the advanced follicles of the surrounding area. The collapse and contraction of the connective tissue would cause the movement of the hair follicles towards the centre of the wound. The lack of melanin pigment in these peripheral hair follicles showed that they had been injured by the treatment. Horse skin exposed to liquid nitrogen cooled copper disc produces white, non-pigmented hairs (Bell and Farrel, 1970).

Rat skin will survive freezing to -10^{o}C but the hair lacks pigmentation (Taylor, 1949). In the present study some of the peripheral hairs were grey and contained some pigmentation. This is thought to be related to the stage of growth at the time of freezing. In the grey hairs some of the melanin granules had survived.

The regenerated epidermal cells contained no melanin pigment. Melanin granule production is a complex process (Scarpelli and Ghiga, 1977) which occurs in specialised cells, the melanocytes. The melanocytes had been destroyed by the treatment and were not capable of regeneration or migration from other areas. The sebaceous glands related to the peripheral hair follicles also regenerated. It is thought that there were two possible modes of sebaceous gland regeneration. The first possibility is that uninjured cells situated near the

basal lamina proliferate to form new sebaceous glands. The second possibility is that precursor cells originated from the active cells of the outer root sheath of growing hair follicles (Montagna, 1962).

The less advanced hair follicles resembled early stages of hair follicle formation. This indicated new hair follicle formation. Regeneration and formation of new hair follicles has been reported after injury in the rat (Buther, 1959), the rabbit (Billingham and Ruseal, 1956) and in man (Kligman and Strauss, 1956) but not in the horse.

CHAPTER 9

ULTRASTRUCTURAL OBSERVATIONS OF THE DEGENERATIVE CHANGES SEEN IN HORSE SKIN UP TO TWENTY FOUR HOURS AFTER CRYOSURGERY.

Introduction

The light microscopic study of the changes in horse skin, after cryosurgery (chapter 8) showed uncomplicated, rapid healing. The most significant observation in the light microscope was the thickness of the epithelium which covered the wound (chapter 8).

During the experimental work tissue was taken for electron microscopy in order to study the effect of cryosurgery in greater detail. For the ease of description, the degenerative and regenerative changes are reported in different chapters.

Observations

A Normal

The epidermis of horse skin was relatively thin (fig. 9.1) and could be divided into four separate regions for descriptive purposes. The four layers were the innermost basal cell layer or stratum germitivum, the stratum spinosum, the stratum granulosum and the outermost stratum corneum (fig. 9.1). The epidermis was separated from the underlying dermis by a basement membrane complex (fig. 9.1, 9.2).

The basement membrane complex consisted of a lamina lucida, which juxtaposed the membrane of the basal cells and an electron dense, amorphous basal lamina (fig. 9.2). Anchoring fibres were inserted into the dermal aspect of

the basal lamina (fig. 9.2). Dermal collagen fibres looped through the loops of the anchoring fibres (fig. 9.2). Between the hemidesmosomes of the basal cell and the basal lamina the lamina lucida contained granular material (fig. 9.2).

The nuclei occupied a very large portion of the basal cells (fig. 9.1). The basal cells contained numerous tonofilaments, which were orientated perpendicular to the surface (fig. 9.2). The tonofilaments inserted into the hemidesmosomes (fig. 9.2).

The basal cells contained numerous melanosomes (fig. 9.1, 9.2). These were membrane bound, ovoid bodies with an average length of 0·7 μ (fig. 9.2). The immature melanosomes (fig.9.2) had an electron dense core surrounded by less electron dense, granular material. The mature melanosomes (fig. 9.2) were uniformly electron dense. Occasionally melanosomes were present in the other cell layers above the basal cell layer (fig. 9.1).

Above the basal layer was the stratum spinosum (fig. 9.3) which consisted of polyhedral cells. The stratum spinosum cells contained abundant cytoplasm packed with tonofilaments (fig. 9.3). The perinuclear region of the stratum spinosum cells contained only ribosomes, mitochondria and occasional melanosomes (fig. 9.3). There were numerous desmosomes along the cell membranes of adjoining stratum spinosum cells (fig. 9.3).

The cells of the stratum granulosum were much flatter than those of the stratum spinosum (fig. 9.4) with their

long axes parallel to the surface. The stratum granulosum is characterised by the appearance of irregular, electron dense, keratohyalin granules (fig. 9.3, 9.4). The keratohyalin granules were found at the periphery of the flattened cells, closely associated with the tonofilament bundles (fig. 9.4). The stratum granulosum was two to three cells thick (fig. 9.3, 9.4).

The surface layer or stratum corneum was composed of very flattened squames which contained no cell organelles (fig. 9.4, 9.5). The stratum corneum cells were fully keratinized and contained keratin fibres embedded in a less dense matrix (fig. 9.5). The stratum corneum was on average 10 cells thick (fig. 9.1, 9.4). All that remained of the desmosomes in the stratum corneum was the central electron dense plaques in the intercellular spaces (fig. 9.5).

The superficial layer of the dermis, immediately underlying the epidermis, contained randomely orientated bundles of collagen, interspersed with fibroblasts, small blood vessels (fig. 9.1) and the occasional mast cell (fig. 9.6).

In the deeper dermis the bundles of collagen were much thicker and there were fewer cells. The skin appendages, the hair follicles, sebaceous glands and sweat glands were situated in the deeper part of this layer.

The deepest part of the hair follicle (fig. 9.7) was the papilla which contained an aggregation of poorly differentiated stem cells calls the matrix. Cells in

the process of mitosis were seen within the matrix (fig. 9.7). Melanosomes were seen in isolated cell processes between the poorly differentiated cells (fig. 9.7). Further out from the centre of the papilla the cells were flatter and contained large, electron dense keratohyalin granules (fig. 9.7).

Towards the surface, the hair follicle consisted of an inner medulla containing elongated cells packed with dense bundles of tonofilaments (fig. 9.8). The nuclei of the medulla cells were also elongated (fig. 9.8). The nuclei and tonofilaments were orientated parallel to the long axis of the hair follicle (fig. 9.8).

Peripheral to the medulla were two layers of cells, the innermost was the cuticle of the hair which interdigitated with the cuticle of the inner root sheath (fig. 9.8). The cells of the hair cuticle were cuboidal with conspicuous nuclei and a few tonofilaments (fig. 9.8). The cells of the inner root sheath were smaller and flatter but with similar cell structure (fig. 9.8).

Adjoining the cuticle of the inner root sheath was the layer of Huxley (fig. 9.8). The cells changed from polyhedral to elongated flattened cells (fig. 9.8). Their cytoplasm was filled with large keratohyalin granules.

The layer of Henle, the outermost layer of the inner root sheath consisted of cuboidal cells (fig. 9.8) and they were flattened and fully keratinised (fig. 9.9).

A connective tissue sheath surrounded the hair follicle (fig. 9.9). It was made up of thick bundles of collagen

and fibroblasts (fig. 9.8, 9.9).

The acinar cells of the sebaceous glands (fig. 9.10) were surrounded by a basal lamina. The connective tissue adjacent to the basal lamina contained numerous fibroblasts and collagen fibres (fig. 9.10). The acinar cells were epithelial cells with desmosomes, centrally positioned nuclei and a few tonofilaments confined to the periphery of the cell (fig. 9.10). The cytoplasm contained numerous droplets (fig. 9.11). In the present preparation the droplets were electron translucent (fig. 9.11). It is thought that the lipid normally present in the acinar cells of the sebaceous glands had been removed during fixation. The mitochondria were also plentiful and active (fig. 9.11).

The sweat glands were of the eccrine type (fig. 9.12). The gland was composed of a layer of columnar epithelial cells surrounding a central lumen (fig. 9.12). The cells contain an abundance of secondary granules of various electron density (fig. 9.12, 9.13). The granules were surrounded by densely packed ribosomes. The surface of the cells facing the lumen was composed of microvilli (fig. 9.13). Peripheral to the secretory cells were the flat myoepithelial cells (fig. 9.12). The secretory tubule was enclosed by a basement membrane and collagen fibres of the connective tissue.

B Twenty minutes after treatment

The first cells to show evidence of injury were the basal cells of the central zone (fig. 9.14). There was contraction of the cells which had created large

intercellular spaces (fig. 9.14). The cytoplasm of the basal cells was vaculated (fig. 9.14) and the nuclei were disturbed. The basal lamina in this region was largely intact, although in some areas it has been ruptured (fig. 9.14).

The strata spinosum and granulosum was also disturbed (fig. 9.15) and the cells showed a loss of cell membrane. Only the desmosomes remained to indicate the boundaries of individual cells (fig. 9.15). The intercellular spaces were filled with granular debris. The cells also contained numerous vacuoles which had the appearance of degenerate mitochondria (fig. 9.15).

The collagen bundles of the underlying connective tissue were more scattered (fig. 9.16) as there was abundant oedematous fluid present. Intact fibroblasts were found along with scattered cell debris (fig. 9.16).

The cell layers of the hair follicle were severely disturbed (fig. 9.17). The only layer that was still defined was the keratinized cells of the layer of Henle (fig. 9.17). Even the fibroblasts of the surrounding connective tissue layer were disrupted and distorted (fig. 9.17).

The basal lamina of the sebaceous glands was still intact (fig. 9.18). Individual cells were easily recognised but the cytoplasmic contents were very degenerate (fig. 9.18). The mitochondria had lost their internal structure (fig. 9.18). The nuclei contained aggregations of dense chromatin material (fig. 9.18).

The lumen of the sweat gland tubules were packed with small vesicles (fig. 9.19). The vesicles were of various shapes and sizes (fig. 9.19). Some of the vesicles had granular or membranous contents (fig. 9.19). The ordered structure of the secretory cells was completely destroyed (fig. 9.19). The nuclei were degenerate but the microvilli could still be recognised (fig. 9.19). The basal lamina and surrounding connective tissue elements were intact and normal (fig. 9.19).

C Twenty four hours after treatment

At twenty four hours the central zone was totally necrotic. The epidermal cells were fatally injured (fig. 9.20) with large intercellular spaces. Desmosomes (fig. 9.20) were still present with a normal appearance. The basal lamina could still be discerned although the associated collagen fibres had totally disintegrated (fig. 9.20). However, the basal lamina was wider and more dispersed than in the normal and twenty minute biopsies (fig. 9.21). Tonofilaments and occasional ribosomes could be seen in the basal cells (fig. 9.21).

The dermis underlying the epidermis of the central zone was also extremely disrupted (fig. 9.22). There were many free erythrocytes dispersed between bundles of collagen and cell debris (fig. 9.22). The collagen fibres were surrounded by dense granular material (fig. 9.22, 9.23) which gave the bundles a hyalinized appearance. Individual collagen fibres had

partially disintegrated (fig. 9.23). The cellular elements of the dermis were ruptured and largely degenerate (fig. 9.22).

In the peripheral regions the intact epidermis and dermis were forming small bullae (fig. 9.24). The contents of epidermal cells were badly affected by the treatment (fig. 9.24) but there had been less separation of the cells from each other. The intercellular spaces in the epidermis were filled with dense, granular material (fig. 9.24). The basal lamina had disintegrated (fig. 9.24). The area immediately adjacent to the epidermis contained floccular oedematous fluid (fig. 9.24). Recognisable cell debris and free erythrocytes were present in the area beneath the floccular oedematous fluid (fig. 9.24).

Also at the periphery of the lesion were many polymorphs (fig. 9.25). The polymorphs were interspersed between the degenerate epithelial cells.

The hair follicles in the central region showed a complete loss of cellular structure (fig. 9.26). Remnants of the layer of Henle were the only recognisable feature (fig. 9.26). The connective tissue layer surrounding the hair follicle was also highly disturbed (fig. 9.26). Polymorphs were present in the surrounding connective tissue (fig. 9.26). Towards the periphery, the cellular nature of the hair follicles could be identified (fig. 9.27) but the cell contents were completely destroyed.

The sebaceous glands in the central region had completely disintegrated by 24 hours after cryosurgery. In the peripheral zone (fig. 9.28) the damage was less severe. However, the basal lamina was no longer present around the majority of the acina (fig. 9.28). The connective tissue cells and the collagen fibres surrounding the sebaceous gland (fig. 9.28) had disintegrated.

The sweat glands were no longer recognisable by 24 hours after treatment.

Discussion

The effect of cryosurgery on horse skin showed progressive degeneration for the first twenty four hours. The early changes seen in the basal cells of the horse skin were similar to those seen in the ventral tongue epithelium by Whittaker (1975) in the hamster.

The cell damage seen at twenty minutes after treatment in the strata spinosum and granulosum was associated with disintegration of the plasma membrane. The causes of such fast and severe degeneration are not fully understood, although the cells were reflecting the effect of freezing and subsequent thaw. The cell membrane of the basal cells was less affected than the strata spinosum and granulosum. The cell membrane of the more superficial cell layers of stratified squamous epithelium is undergoing change and development, during the process of keratinization. The changes in the cell membranes

include the deposition of the contents of the membrane coating granules on the cell surface (Matolsty and Parakka, 1965). This could well mean that the cells became more susceptible to the effect of cryosurgery.

The strata spinosum and granulosum cells contain a higher proportion of cytoplasm than the basal cells. Consequently the numbers of ice crystals present in the more superficial layers may have been greater and more damaging than in the basal cells.

The accumulation of granular material in the intercellular spaces was thought to be cell debris which had escaped from the cells with damaged cell membranes.

By twenty four hours after treatment the epidermal cells of the central region showed total necrosis. The blisters which had developed at the periphery of the lesion were similar to those seen in human skin after sunburn (Graham, 1971). However, the epithelial cells of the horse skin blister showed more distortion after cryosurgery than those created by other means; for example blisters caused by suction (Kiistala and Mustakallio, 1967). Pearson (1964) noted that prolonged freezing with liquid nitrogen resulted in blister formation with a distinct dermal-epithelial separation. The separation occurred as a widening of the lamina lucida (Pearson, 1964). Pearson (1964) suggested that the basement membrane complex was more adversely altered by freezing than the specialised intercellular junctions of the dermis. The observations in the present study on horse skin support this view but

it is interesting to note that in the study on muscle and peripheral nerve, the basement membrane complex was one of the most resistant tissue components.

In the early stages after treatment the desmosomes and hemidesmosomes showed resistance to cold treatment. However, with time there was a progressive reduction in the numbers of desmosomes and hemidesmosomes. It is thought that the reduction of desmosome and hemidesmosome numbers was related to the amount of oedematous fluid containing polymorphs and other inflammatory cells. Graham (1971) showed that the inflammatory cells in damaged skin liberated proteolytic enzymes which could effect the desmosomes and hemidesmosomes.

The earlier studies on skeletal muscle and peripheral nerve showed the collagen fibres to be very resistant to low temperature. This was not the case in the skin where the collagen fibres were in the process of disintegration and hyalinization by twenty four hours after treatment. In vivo studies on human skin have shown collagen fibre degeneration by 72 hours after a cotton swab soaked in liquid nitrogen had been applied (Zacarian, 1969). The difference in time scale was most probably due to the relative effectiveness of the two methods of applying cold treatment.

The observations on the effect of cryosurgery on collagen in the three tissues reflects the differences in basic collagen fibre chemistry. It is interesting to speculate on the role of oedematous fluid and inflammatory

cells on the degeneration of the collagen bundles and fibres.

The damage to the hair follicles in the central region was consistant with the effects of freezing and thawing. The keratinized cells, containing little water, were less injured than the cells at an earlier stage of differentiation.

The cells of the sebaceous and sweat glands were disrupted by the treatment with the sweat glands showing the greatest sensitivity to cold. The accumulation of membrane bound vesicles in the lumen of the sweat glands was probably a result of damaged microvilli. The microvilli of hepatic cells were the most sensitive region after *in vitro* freezing (Trump *et al.*, 1964).

CHAPTER 10

OBSERVATIONS ON THE REGENERATIVE CHANGES IN HORSE SKIN AFTER CRYOSURGERY

Introduction

By 24 hours after cryosurgery there was destruction of the epidermis and liquifaction degeneration of the dermis, with an inflammatory infiltrate of blood cells (chapter 9). One interesting feature of the effect of cryosurgery on horse skin was the disintegration of the collagen bundles of the dermis (chapter 9). The collagen fibres of muscle and nerve tissue, observed in this study, were very resistant to low temperatures (chapters 3 and 6).

This chapter will report the electron microscope observations of horse skin, one, two, three and four weeks after treatment.

Materials and Methods.

i) Surgical procedure see chapter 8, materials and methods section i).

ii) Electron microscopy see chapter 3, materials and methods section iii).

Observations

A One week after treatment

The centremost part of the lesion was full of necrotic debris containing only a few recognisable structures, for example, erythrocytes, degenerate nuclei and bundles of collagen fibres (fig. 10.1). Distributed along the collagen bundles were electron dense flecks (fig. 10.1).

These flecks were orientated with their long axes parallel to the long axes of the collagen fibres (fig. 10.1). In some bundles the collagen could be recognised as discrete fibres (fig. 10.1, 10.2) but other bundles of collagen fibres had a hyalin appearance (fig. 10.1, 10.2).

Peripheral to the centremost areas, numerous polymorphs were scattered amongst the debris and degenerate cells (fig. 10.2). The hair follicles in this region were also highly degenerate (fig. 10.3) and were only recognised by the electron dense debris confined within the basal lamina of the follicle.

The most significant change observed at one week post operatively was the proliferation of the epidermis towards the centre of the lesion. There were several mitotic figures (fig. 10.4) in the centremost part of epidermal proliferation. The newly formed epidermal cells contained conspicuous nuclei and a relatively small proportion of cytoplasm (fig. 10.4). The desmosomes of these newly formed cells were sparse (fig. 10.4). The cell membranes of juxtaposed cells were firmly pressed together with no intercellular spaces (fig. 10.4). This was particularly conspicuous in a region (fig. 10.5) where newly formed cells abutted existing epidermal cells. The newly formed cells showed fewer desmosomes, larger nuclei and fewer tonofilaments than the original cells (fig. 10.5). The newly regenerated cells contained rough endoplasmic reticulum cysternae, not seen in normal epidermal cells (fig. 10.4, 10.5). There was

a complete absence of melanosomes in the regenerated epidermal cells (fig. 10.4, 10.5).

The hemidesmosomes of the basal cell membranes in the regenerating epidermis (fig. 10.6) were not well established even though a basal lamina was present. The hemidesmosomes showed various stages of development from just thickenings of the cell membrane (fig. 10.7) to formation of the lamina lucida component (fig. 10.7). The desmosomes were also at a developmental stage (fig. 10.7). In some areas potential sites for desmosome formation were indicated by the close apposition of adjacent cell membranes (fig. 10.7). In other areas (fig. 10.7) early stages of attachment plaque formation was seen as accumulations of electron dense material just under the cell membrane.

The collagen fibres immediately beneath the basal lamina of the regenerate epidermis, were randomely orientated (fig. 10.6, 10.7). Slightly deeper into the dermis the collagen fibres were organised into bundles (fig. 10.6). This region of the dermis contained active fibroblasts (fig. 10.6) and occasional egg-shaped cells. The egg shaped cells were heavily surrounded by closely associated collagen fibres (fig. 10.6). This cell (fig. 10.6) was packed with fine fibrils but contained only sparse organelles.

The stratum spinosum cells (fig. 10.8) in the regenerating region were packed with tonofilament bundles. The intercellular spaces were not as large or conspicuous

as those in the normal epidermis (fig. 10.8).

The skin appendages could still be recognised in the peripheral regions. Growth was still occurring in the hair follicles in the peripheral region as mitotic figures were present in Henle's layer (fig. 10.9). Melanosomes were still present in the medulla cells (fig. 10.9).

The sebaceous glands in the peripheral region showed little disturbance (fig. 10.10). On the other hand the sweat glands in the same area were highly degenerate (fig. 10.11).

B Two weeks after treatment

The centre of the lesion was covered with a dense scab (fig. 10.12, 10.13). Part of the scab contained remnants of fibroblasts and other cells embedded in a dense, amorphous matrix (fig. 10.12). Another region showed scattered erythrocytes embedded in the same amorphous matrix (fig. 10.13). In the deeper regions of the scab were bundles of collagen fibres also surrounded by the amorphous matrix (fig. 10.13).

Normal epidermal cells had penetrated under the peripheral edge of the scab (fig. 10.14). The stratum corneum of the regenerated epithelium was only two cells thick (fig. 10.14) and the stratum granulosum was not fully established (fig. 10.14). The stratum granulosum was only one cell layer thick (fig. 10.14) and the cells were not as flattened as in the normal skin. The keratohyalin granules were more scattered throughout the cytoplasm (fig. 10.14). The stratum corneum cells were

flattened, electron dense and their cell membranes interdigitated with one another (fig. 10.14). The stratum spinosum cells had a normal appearance (fig. 10.14, 10.15). The intercellular spaces in the basal region of the regenerated epidermis contained various vacuoles embedded in electron dense material (fig. 10.15). There were several non-epithelial cells present in the newly formed epidermis (fig. 10.15). These cells were either lymphocytes (fig. 10.15) or polymorphonuclear leucocytes (fig. 10.16).

The basement membrane complex in this region was normal (fig. 10.15).

There were no hair follicles in the central zone but in the peripheral regions the hair follicles were active (fig. 10.17). The hair follicle cells were normal and healthy (fig. 10.17) which contrasted with the neighbouring sebaceous gland cells. The sebaceous gland cells contained vaculated and degenerate cytoplasm (fig. 10.17). There were no melanosomes in any of the cell layers (fig. 10.17). The layer of Henle was fully keratinized (fig. 10.17).

The most significant feature of the peripheral hair follicles was the absence of cuticle layers (fig. 10.17). The medulla cells were immediately juxtaposed to the cells of Huxley's layer (fig. 10.17). The cells of Huxley's layer contained tonofilaments (fig. 10.18, 10.17) and clusters of electron dense granules. Each cell contained several clusters of granules (fig. 10.18). The

granules were not membrane bound and were of varying degrees of electron density (fig. 10.18).

There were signs of sebaceous gland regeneration in the peripheral regions (fig. 10.19). The sebaceous gland cells were active and contained many secretory droplets (fig. 10.19).

It was difficult to know whether the sweat glands seen at the very periphery of the lesion were regenerated or had withstood the treatment (fig. 10.20).

C Three weeks after treatment

The epidermis continued to cover the surface of the wound. One of the most striking features of the epidermis in the centre of the lesion was the mass of connective tissue cells in the area immediately beneath the basal lamina (fig. 10.21). The epidermal, basal cells contained only a few tonofilaments, hemidesmosomes and desmosomes (fig. 10.21). The majority of the connective tissue cells contained prominent nuclei and only sparse cytoplasm (fig.10.21). It was difficult to distinguish particular cell types amongst the mass of cells (fig. 10.21).

The stratum corneum of the more peripheral, regenerated epidermis had increased to four to six cells thick (fig. 10.22). The number of cells in the stratum granulosum had also increased (fig. 10.22). The stratum granulosum cells contained large membrane bound vacuoles (fig. 10.22). These were thought to be swollen degenerate mitochnodria. There were numerous membrane coated granules in the upper stratum spinosum cells (fig. 10.22). Membrane

coated granules were not conspicuous in the normal skin (chapter 9).

Away from the very centre of the lesion the dermis immediately beneath the epidermis consisted of collagen fibres and fibroblasts (fig. 10.23). The basal lamina was not fully formed and there were only a few hemidesmosomes in each basal cell (fig. 10.23). Tonofilaments were seen associated with the attachment plaques of some hemidesmosomes (fig. 10.24). In other regions of the cell the hemidesmosomes were represented by a thickened region of the cell membrane (fig. 10.24). The basal lamina was incomplete (fig. 10.24) and had the same texture as the connective tissue matrix. The basal lamina was just more electron dense than the surrounding matrix (fig. 10.24). The basal cells contained small, circular vesicles which were situated close to the cell membrane (fig. 10.24).

The skin appendages in the peripheral region had the same appearance as those at two weeks after treatment.

D Four weeks after treatment

In the centremost part of the lesion the stratum corneum was thin and not fully keratinized (fig. 10.25). The stratum corneum cells still contained ribosomes and intact desmosomes (fig. 10.25). There were lipid droplets scattered amongst the tonofilament bundles in the stratum corneum cells (fig. 10.25). The underlying stratum granulosum cells were not flattened and contained only a few keratohyalin granules (fig. 10.25). The perinuclear cytoplasm of the stratum granulosum cell contained

large vacuoles (fig. 10.25).

Towards the periphery of the central region the epidermis had a normal appearance (fig. 10.26). The stratum granulosum contained conspicuous keratohyalin granules and the stratum corneum cells were fully keratinized (fig. 10.26). The basal cell layer of this region was also normal (fig. 10.27). The hemidesmosomes and desmosomes were fully formed (fig. 10.27). The basal lamina was not completely formed but the collagen fibres in the underlying connective tissue were normal (fig. 10.27).

There were clumps of electron dense debris in the connective tissues of the centre of the biopsy (fig. 10.28). The electron dense debris had the appearance of a partially digested keratinized cell (fig. 10.28). In the peripheral regions the connective tissue underlying the epidermis contained active fibroblasts, macrophages and small blood vessels (fig. 10.29). Scattered between the cells were collagen fibres and vacuoles of various sizes containing debris.

The skin appendages in the peripheral regions had a similar appearance to those at two weeks after treatment.

Discussion

In this chapter, the observations were mainly undertaken to study the cellular response during the regenerative period after an induced cryolesion in horse skin.

One week after treatment the centre of the lesion showed total necrosis and the normal tissue had been replaced by a scab. The scab did contain some cellular elements which is in contrast to Ordman and Gillman's (1966) results, in which there were no cellular elements in the scab. The observations showed no specific attachment mechanism between the scab and the adjacent regenerating epithelium. There was just an amorphous area separating the damaged central area from the regenerating epidermis.

The main source of cells for the regenerating epidermis was from the intact basal cells at the wound margin (Montagna, 1962; Schilling, 1968). The epithelial metaplasma was remarkable at the wound edges in the horse skin. This metaplasia was not seen in the tonsil of the dog after cryosurgery (Hall, Jackson and Turner, 1969). The degree of epithelial thickening was related to the amount of necrotic tissue. Croft and Tarin (1970) studying excised skin showed that regenerating epithelium could not migrate across necrotic tissue but only accumulate around the wound edges.

The stages of hemidesmosome and desmosome morphogenesis showed the same developmental events as those seen in mouse skin after trauma (Krawczk and Wilgram, 1973).

The relative roles of epidermal basal cells and the

connective tissue in basement membrane formation has long been subject to controversy. The early workers thought that connective tissue was solely responsible for basement membrane production. More recent studies have shown that epithelial cells do contribute to basement membrane production (Hay and Revel, 1963; Odland and Ross, 1968; Scinbba, 1977; Van Ness and Simpkins, 1979). In this study orientation and differentiation of the epidermal cells occurred before basal lamina formation.

By the second week, the regenerated epidermis showed early stages of differentiation into different strata. The differentiation was most clearly seen at the periphery. Nearer the centre there was a stratum granulosum and an indication of a stratum corneum. By three weeks the differentiation within the regenerating epidermis was more pronounced.

There were severel non-epithelial cells amongst the regenerating epidermal cells. These lymphocytes and polymorphs were thought to be related to a defense mechanism against pathogens. An incomplete epidermis would be more susceptible to pathogen attack. Also an incomplete basal lamina would allow non-epithelial substances and cells to penetrate the epidermis.

The membrane coating granules appeared in large numbers at three weeks after treatment. The membrane coating granules have been shown to be involved with permeability of epithelia (Squier and Johnson, 1975). The presence of membrane coating granules is associated with

the formation of a barrier to the free movement of substances through the intercellular spaces of the epithelium (Squier and Johnson, 1975). In a regenerating epithelium the appearance of membrane coating granules could well indicate a stabilising and maturing phase in the repair process.

By the fourth week the regenerated epidermis was well differentiated but thicker than the normal There were no melanosomes in the regenerated epidermis.

The dermis and dermal adnexa were necrotic by the first week after treatment. Most of the collagen fibres had been hyalinized. The central necrotic area was surrounded by numerous fibroblasts and inflammatory cells. It has been shown (Grillo, 1963) that the fibroblasts concerned with dermal repair originate from the perivascular mesenchyme. The increase in numbers of fibroblasts could be related to the numbers of inflammatory cells. Ross (1975) showed that the proliferative activity of fibroblasts was affected by the presence of macrophages.

The cells which had accumulated beneath the basal lamina were histiocytes. The histiocytes may have been derived from fibroblasts or lymphocytes (Montagna, 1971) and were usually seen when there was damage to the dermal adnexa.

One week after treatment the hair follicle cells showed evidence of growth and mitosis. At one week there were a few melanosomes in the medullary cells but by the second week they had disappeared. Zacarian (1969) showed

melanocytes to be very sensitive to cold and they die selectively within the hair follicle.

The lack of hair cuticle in the hair follicle was thought to be a result of the cold injury and not to seasonal hair growth variations. The medulla disappears from the hair of equidae as a result of seasonal growth variations (Talukar, Calhoun and Stinson, 1972).

The electron dense granules in the cytoplasm of Huxley's layer cells were thought to be keratohyalin granules. They have the same appearance as keratohyalin granules in the rat buccal mucosa (Kempson, 1976). It is interesting to speculate as to whether the keratohyalin granules in the treated biopsy had a different chemical composition to the normal.

There have been no studies on the regeneration of sebaceous glands in the horse skin after trauma. After injury with methylcholanthrene sebaceous glands in mouse skin will regenerate within one week (Montagna, 1971). The regenerating sebaceous cells were derived from the outer root sheath of surviving hair follicles (Montagna, 1971). In the present study regenerated sebaceous glands were associated with the presence of intact hair follicles.

There was no evidence to indicate that sweat glands could regenerate during the period of the experiment. The sweat glands were the most sensitive structure in the skin to cold treatment.

The skin and its adnexa is a complex structure.

The present study into the effect of cryosurgery on horse skin has stimulated more questions than it has solved. A much greater in depth study is required to fully elucidate the healing process after cryosurgery.

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